A COMPARISON OF THIAMINE SYNTHESIS AND EXCRETION IN HUMAN SUBJECTS ON SYNTHETIC AND NATURAL DIETS

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The study of Najjar and Holt ('43) on the biosynthesis of thiamine in man stimulated interest in such synthesis as a source of B vitamins in human nutrition. The suggestion was made in Nutrition Reviews ('44) that the specific dietary regimen used by these workers might be unusually conducive to bacterial synthesis in the intestinal tract. The present study was undertaken to compare the thiamine excretion of subjects on a synthetic diet with their excretion on a controlled diet of natural foods of similar nutritive value.

EXPERIMENTAL

Subjects. Three normal women acted as subjects for the experiment. Subjects A, B, and C were 21, 31, and 46 years of age, and their weights during the experimental periods were about 128, 133, and 138 pounds, respectively. Their respective heights were 63, 66, and 67 inches. Physical examinations of the subjects, made at the University Clinic at the beginning and end of the period on the synthetic diet, indicated that all were in general good health. During the study all of the subjects continued their usual activities, which included only very moderate exercise.

Diets. The synthetic diet was essentially that of Najjar and Holt ('43). It contained 72 gm casein,[1] 128 gm Crisco,

[1] Sheffield Farms low-vitamin casein.

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200 gm Dextri-Maltose,[2] 90 gm distilled water, and 20 gm salt mixture[3] per day. It supplied approximately 2240 cal. A vitamin mixture,[4] prepared in 2% acetic acid, supplied per day 1 mg thiamine, 1 mg riboflavin, 1 mg pyridoxine, 1 mg inositol, 1 mg p-aminobenzoic acid, 5 mg calcium pantothenate, 5 mg choline, and 15 mg niacin. The casein also contained 0.09 mg riboflavin per day. Each meal consisted of one-third of the diet and one-third of the vitamin mixture. One cup of black coffee was allowed with each meal. Two drops of percomorph liver oil,[5] supplying about 5000 USP units of vitamin A and 550 units of vitamin D, and 50 mg ascorbic acid[4] were given with breakfast. The subjects were maintained on this synthetic diet for 7 weeks.

After a month's respite, the following natural diet was used for a period of 3 weeks: 50 gm egg, 100 gm milk, 50 gm cheese,[6] 100 gm ground beef chuck, 20 gm cooked bacon, 100 gm boiled potatoes, 100 gm fresh carrots, 100 gm frozen green beans, 30 gm lettuce, 100 gm tomato juice, 100 gm frozen peaches, 150 gm frozen applesauce, 35 gm raisins, 30 gm cornflakes, 30 gm cooked rice, 26 gm Ry-Krisp, 50 gm peanuts, 15 gm mayonnaise, 35 gm butter, 20 gm brown sugar, and 24 gm peppermint candy per day. This diet was calculated to have approximately the same food value as the synthetic diet, but was found on analysis to contain an average of 0.84 mg thiamine, 0.16 mg less than the synthetic diet.

Collection and care of samples. Twenty-four-hour urine specimens were preserved in brown bottles containing enough glacial acetic acid to maintain an acid concentration of 2 to

[2] Special vitamin-free Dextri-Maltose, supplied through the courtesy of Mead Johnson and Co.

[3] Twenty gm of salt mixture contained 1.75 gm $CaHPO_4$, 0.80 gm $CaCO_3$, 1.33 gm KH_2PO_4, 4.00 gm $KHCO_3$, 1.50 gm KCl, 2.50 gm $Na_2HPO_4 \cdot 7H_2O$, 7.00 gm NaCl, 1.00 gm $MgCO_3$, 0.10 gm $FeSO_4 \cdot 7H_2O$, 0.01 gm $MnSO_4 \cdot H_2O$, 0.005 gm $CuSO_4$, 0.005 gm $ZnSO_4$, and 0.00015 gm KI.

[4] B vitamins and ascorbic acid supplied through the courtesy of Hoffmann-La Roche, Inc.

[5] Abbott.

[6] Cheese supplied through the courtesy of Kraft Cheese Corp.

3%. Samples were stored under refrigeration, and were analyzed within 72 hours. Feces were marked with carmine. Individual feces specimens were made 10% acid by addition of glacial acetic acid, and preserved under refrigeration in 5-day composites. During 4 days of the study on the natural diet, samples of the foods as eaten were collected at each meal, immediately macerated in a Waring blendor, and made to volume. Aliquots were quick frozen for later analysis.

Methods of analysis. The thiamine content of urine, feces, and foods was measured by the modification by Najjar and Ketrone ('44) of the Hennessy thiochrome method ('42). Feces were examined for both "free" and "total" thiamine, i.e., before and after samples had been incubated with Mylase P.[1]

RESULTS AND DISCUSSION

Urinary excretion of thiamine. The values for the average daily intake and excretion of thiamine by the three subjects are given by 5-day periods, table 1. The daily urinary excretion values on the synthetic diet were, for subject A, 116 ± 17.9 μg, for subject B, 113 ± 20.0 μg, and for subject C, 147 ± 18.1 μg. On the natural diet the corresponding values were 90 ± 8.7 μg, 91 ± 17.5 μg, and 112 ± 20.3 μg. The lower values on the natural diet reflect the lower intake of thiamine on this diet (a reduction of 160 μg per day). The 5-day averages were relatively constant for each individual, but subject C excreted an average of 31 to 34 μg more than the other subjects while on the synthetic diet, and 21 to 22 μg more while on the natural diet. Since subject C weighed 10 and 5 pounds more than subjects A and B, respectively, and the food intake of all subjects was the same, this difference in excretion is not related to body weight or food intake.

These urinary excretion values are similar to those reported in the literature for subjects on comparable thiamine intakes. The fact that on the synthetic diet there is no tendency for decreased urinary excretion might indicate that the level of

[1] Wallerstein Laboratories.

TABLE 1

Average daily excretion of thiamine by three women.

PERIOD	SUBJECT A				B				C			
	Urine: Free	Feces		Total	Urine: Free	Feces		Total	Urine: Free	Feces		Total
		Free	Combined			Free	Combined			Free	Combined	
	μg	μg	μg	μg	μg	μg	μg	μg	μg	μg	μg	μg
Synthetic diet containing 1.00 mg thiamine per day												
1	99	..	...	...	97	..	..	...	158	..	..	...
2	122	18	121	261	142	15	37	194	178	13	93	284
3	87	17	107	211	95	16	35	146	138	15	49	202
4	107	9	112	228	109	19	91	219	135	15	133	283
5	112	19	127	258	106	10	42	158	138	2	30	170
6	120	14	70	204	109	17	29	155	138	31	77	246
7	127	23	90	240	106	6	21	133	148	12	106	266
8	128	28	119	275	120	20	62	202	151	9	89	249
9	138	7	43	188	137	20	63	220	142	8	73	223
Av.	116	17	99	233 [1]	113	15	48	178 [1]	147	13	81	240 [1]
Natural diet containing 0.84 mg thiamine per day												
1	95	30	268	393	109	43	292	444	137	30	386	553
2	91	22	184	297	99	53	336	488	117	23	268	408
3	84	12	271	367	78	47	145	270	93	30	535	658
4	90	38	229	357	78	54	122	254	100	55	272	427
Av.	90	25	238	353	91	49	244	364	112	35	365	512

[1] Period 1 omitted in computing total excretions.

1.0 mg per day was adequate for maintenance of thiamine needs for these subjects. The decrease in urinary thiamine excretion for subjects B and C on the intake of 0.84 mg might indicate that this level of intake is lower than their customary ingestion of the vitamin. The excretion might have decreased further had the study been continued.

Fecal synthesis and excretion of thiamine. The values for the average daily fecal excretion of thiamine by periods are included in table 1. On the synthetic diet general averages for "free" thiamine for eight 5-day periods were 17, 15, and 13 μg, values far below those found by Najjar and Holt ('43) in subjects who were free from deficiency symptoms when they were on thiamine-free diets. On the natural diet the average "free" thiamine excretions were increased to 25, 49, and 35 μg per day. The values for "combined" thiamine (total thiamine minus "free" thiamine) on the diet of natural foods were 2.4, 5.1, and 4.5 times greater than the values on the synthetic diet.

The "combined" thiamine is generally thought to lie within the bacterial cells, and to be unavailable for assimilation. Alexander and Landwehr ('45) contend that no thiamine is absorbed from the colon, and consequently that the free thiamine as well as the combined is unavailable. This conclusion is based on a comparison of the urinary and fecal thiamine excretion of one subject on 2 successive days, the second day following a retention enema containing added thiamine and cocarboxylase. From the daily variations in urinary thiamine excretion found in the subjects of the present study and the difficulty in marking 24-hour stools, it seems unjustifiable to base such a conclusion on the evidence of one set of tests on one subject.

The fact that less thiamine was excreted in the feces on the synthetic diet than on the natural diet might indicate that the conditions on the synthetic diet were less favorable for bacterial synthesis of thiamine than those on the natural diet. The facts that the urinary thiamine excretions on the natural diet reflected the lowered thiamine intake, and that the in-

creases in fecal excretion of free thiamine were small, might indicate that even on the natural diet fecal synthesis of thiamine is not an important factor in the thiamine economy of these three subjects.

Significance of the results in relation to human requirement for thiamine. In recent reviews of the experimental data available as a basis for the determination of the thiamine requirement of man, Melnick ('44) concludes that 0.6 mg per 1000 cal is not an excessive allowance, while Holt ('44) considers that between 0.17 and 0.23 mg per 1000 cal is the basic requirement. These values probably represent the extremes between which the average requirement will be found. In the revised recommended dietary allowances of the National Research Council ('45), the thiamine allowance for women has been set at 1.1 to 1.2 mg.

As a result of recent extensive studies on men having a restricted intake of B vitamins, Keys and associates ('45) conclude that clinical impressions do not give conclusive evidence of thiamine deficiencies. Although urinary thiamine excretions are probably significant, the level of excretion which indicates adequate thiamine metabolism is still questionable. They also question whether the thiamine requirement is actually strictly and linearly proportional to calories, or even to the non-fat calories, and suggest that man may require a constant amount, plus an amount related to total or non-fat calories. From these and other data in the literature (Jansen, '32; Van Veen, '40; Elsom et al., '42) it appears probable that 0.6 to 0.7 mg per day will prevent symptoms of deficiency for at least a month, and that 1.0 mg will allow a small margin of safety in healthy persons for 6 months or perhaps indefinitely.

The thiamine intakes of subjects in the present study (0.84 to 1.00 mg per day, or 0.37 to .45 mg per 1000) lie between the limits suggested above. No symptoms of deficiency were evident on the lower level of intake, but the daily urinary thiamine excretion fell below 100 μg for two of the subjects. These intakes appear to have been adequate for the periods

during which they were used, and the results of the study support the evidence of other workers that lowering the recommended daily allowance for women to 1.1 or 1.2 mg per day gives an adequate margin of safety under normal conditions.

SUMMARY AND CONCLUSIONS

Three normal women were maintained on a synthetic diet containing 1.00 mg of thiamine per day for 7 weeks. This diet was followed, after a month's respite, by a diet of natural foods containing 0.84 mg of thiamine per day. Excretion of thiamine in the urine was measured daily, and in the feces by 5-day periods. The results may be summarized as follows:

1. Daily urinary thiamine values averaged 116, 113, and 147 μg on the synthetic diet, and 90, 91, and 112 μg on the natural diet.

2. The average values for daily excretion of "free" thiamine in the feces were 17, 15, and 13 μg on the synthetic diet, and 25, 49, and 35 μg on the natural diet.

3. "Combined" thiamine of the feces was 2.4, 5.1, and 4.5 times higher on the natural than on the synthetic diet, suggesting that the synthetic diet was less favorable for bacterial synthesis of thiamine than the natural diet.

4. The facts that urinary thiamine excretions on the natural diet reflected the lowered thiamine intake and that increases in fecal excretion of "free" thiamine were small, might indicate that fecal synthesis of thiamine was not an important factor in the thiamine economy of these three subjects.

5. The excretions of thiamine by these subjects and their apparent well-being on thiamine intakes of 0.84 to 1.00 mg support the lowering of the recommended daily allowance for women to 1.1 or 1.2 mg per day.

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A COMPARISON OF RIBOFLAVIN SYNTHESIS AND EXCRETION IN HUMAN SUBJECTS ON SYNTHETIC AND NATURAL DIETS

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The observation by Najjar and Holt ('43) of the biosynthesis of thiamine in man, stimulated interest in fecal synthesis as a source of other vitamins of the B complex. In 1944 Najjar and coworkers reported synthesis of riboflavin in man. Data are presented in the present study on synthesis and excretion of riboflavin by three women on the synthetic diet of Najjar and Holt ('43) and on a diet of natural foods of similar nutritive value. Details concerning the subjects, diets, and collection and preservation of samples are given in the report by Hathaway and Strom ('46).

The riboflavin content of the samples was determined by the method of Najjar ('41).

RESULTS AND DISCUSSION

It is difficult to plan a diet adequate in other dietary essentials which contains as little as 1.0 mg of riboflavin. In this study the riboflavin content of the natural diet proved on analysis to be higher than had been calculated. The synthetic diet contained 1.09 mg per day of riboflavin, or 0.48 mg per 1000 cal, the natural diet 1.33 mg per day or 0.59 mg per 1000 cal. These differences in riboflavin intake complicate the comparison of the urinary excretions of the subjects on the two diets, but probably do not alter the fecal excretions.

Davis ('45) found fecal excretions in five subjects unaltered by variations in riboflavin intake.

Urinary excretion of riboflavin. The average daily intake and excretion of riboflavin by these three subjects are reported by 5-day periods in table 1.

On the synthetic diet the average urinary excretion of riboflavin decreased markedly until the end of the third period, suggesting that the normal riboflavin intake of these subjects had been greater than the 1.09 mg in the synthetic diet. By period 4 the values for subjects B and C had approached a plateau, but single high values (over 230 μg) during periods 4 and 5 held these 5-day averages for subject A above her average for the last 6 periods. Daily urinary excretions for periods 4 through 9 were 165 ± 25.9 μg, 152 ± 19.2 μg, and 161 ± 24.1 μg for subjects A, B, and C, respectively. The urinary riboflavin excretions were greater on the natural diet than on the synthetic diet but by period 2 had reached a plateau for subjects A and C. They were still high for subject B. Excretions during the last three periods were, for subject A, 174 ± 35.4 μg, for subject B, 229 ± 48.7 μg, and for subject C, 210 ± 35.6 μg.

It is interesting to compare these data with those from a preliminary experiment planned primarily to study thiamine metabolism. Subject B took part in this study, in which a different diet of natural foods was used (Gifft, '44). Her riboflavin intake was 1.40 mg per day, or 0.54 mg per 1000 cal. During the first 2 weeks daily determinations of urinary riboflavin were made, and for the last 4½ weeks, analyses were made of two 3-day aliquots per week. Values for these last nine determinations were 252 ± 40.6 μg. Corresponding values for a second woman on the same level of intake were 269 ± 39.1 μg. Two men also acted as subjects for this study. Their riboflavin intakes were higher than for the women, one subject on an intake of 1.49 mg or 0.48 mg/1000 cal, excreted 266 ± 35.5 μg, and the other on an intake of 1.56 mg or 0.44 mg/1000 cal, excreted 382 ± 31.6 μg. The riboflavin ex-

TABLE 1

Average daily excretion of riboflavin.

PERIOD	SUBJECT A				SUBJECT B				SUBJECT C			
	Urine: Free	Feces		Total	Urine: Free	Feces		Total	Urine: Free	Feces		Total
		Free	Combined			Free	Combined			Free	Combined	
	μg	μg	μg	μg	μg	μg	μg	μg	μg	μg	μg	μg
	Synthetic diet containing 1.09 mg riboflavin per day											
1	269	...	...	...	311	...	...	...	478	...	...	...
2	267	317	170	754	308	123	44	475	375	237	192	804
3	203	332	188	723	224	178	136	538	239	300	187	726
4	196	298	115	609	169	218	99	486	179	387	184	750
5	177	325	98	600	144	125	40	309	182	113	59	354
6	155	153	59	367	146	154	76	376	170	288	166	624
7	157	273	62	492	146	58	22	226	146	286	168	600
8	144	...	...	...	153	...	...	...	159	...	...	...
9	162	156	97	415	152	222	92	466	132	165	88	385
Av. 4–9	165	241	86	497 [1]	152	155	66	373 [1]	161	248	133	543 [1]
	Natural diet containing 1.33 mg riboflavin per day											
1	197	616	530	1343	321	759	310	1390	333	697	579	1609
2	174	642	406	1222	273	643	199	1115	234	510	464	1208
3	161	584	646	1391	194	360	297	851	167	997	649	1813
4	187	872	494	1553	219	695	307	1221	227	718	589	1534
Av. 2–4	174	699	515	1389	229	566	268	1062	210	742	567	1518

[1] Period 8 omitted in computing average excretions, periods 4 to 9.

cretions on these higher intakes showed more relation to total intake than to the intake per 1000 cal.

On diets of widely different composition but with a relatively narrow range of riboflavin content (1.0 ± 0.1 mg), the following values have been reported for 24-hour urinary excretions of riboflavin: Williams et al. ('43) 48 to 212 μg; Foltz et al. ('44) 120 to 310 μg; Keys et al. ('45) 113 to 155 μg; Davis ('45) 92 to 210 μg, averaging 150 μg. The riboflavin excretions for each subject averaged 100 μg or more. In the present study, during the last six 5-day periods on the riboflavin intake of 1.09 mg, the urinary excretion values varied from 108 to 240 μg, averaging 152 to 165 μg. These values are in good agreement with the ones reported above.

On the riboflavin intake of 1.33 mg per day, the 24-hour urinary excretions averaged 174 to 229 for the last three 5-day periods. One subject in the Williams et al. study ('43) with intakes at this level, excreted 138 to 156 μg in 3 tests. Davis ('45) reports excretion values for 4 women at about this level of intake, of from 188 to 486 μg, averaging 274 μg for 5 weeks. Thus the excretions at this level are greater than at 1.0 mg, and averages vary more than at the lower level of intake.

Table 2 includes riboflavin excretion values for 1-hour fasting urine specimens collected during 15 days of the study on a daily intake of 1.09 mg of riboflavin. The values varied from 3.7 to 10.9 μg per hour, with average values for the three subjects of 5.9, 7.1, and 7.8 μg. These correspond to the ones reported for Davis' subjects ('45) on intakes of approximately 0.60 mg rather than those on intakes of 1.02 mg. They are in the range reported by Najjar et al. ('44) for 12 young boys on diets containing only 70 to 90 μg per day.

Evidence is presented by Feder, Lewis and Alden ('44) that the riboflavin excretion per milliliter of urine is a more constant value than the excretion per day or per hour. Contrary evidence is presented in table 2. The relationship of the urinary riboflavin excretion to the urinary volume, both on 24-hour and on 1-hour collections, is given for 15 successive

TABLE 2

Relationship of urinary excretion of riboflavin to urinary volume.

DATE	SUBJECT A				B				C			
	24-hour		1-hour		24-hour		1-hour		24-hour		1-hour	
	Total	Per ml	Total	Per ml	Total	Per ml	Total	Per ml	Total	Per ml	Total	Per ml
Nov.	μg	μg	μg	μg	μg	μg	μg	μg	μg	μg	μg	μg
20	192	0.124	...	...	156	0.109	10.1	0.037	175	0.113		
21	198	0.151	7.0	0.032	166	0.130	4.8	0.080	153	0.094	6.2	0.072
22	164	0.106	7.2	0.041	150	0.091	9.9	0.026	186	0.117	6.7	0.099
23	231	0.149	6.0	0.055	199	0.124	9.0	0.032	204	0.116	8.8	0.042
24	184	0.145	4.4	0.045	131	0.102	6.0	0.059	198	0.099	10.8	0.031
25	143	0.126	4.4	0.050	127	0.106	...	....	168	0.088	6.5	0.040
26	181	0.142	10.9	0.094	141	0.117	8.6	0.038	181	0.095	7.7	0.033
27	146	0.108	...	...	123	0.088	5.8	0.065	160	0.099	...	....
28	151	0.086	4.3	0.069	156	0.107	7.8	0.030	170	0.102	7.6	0.044
29	141	0.098	5.6	0.041	152	0.115	6.2	0.050	160	0.078	8.3	0.032
30	155	0.086	4.0	0.087	137	0.119	5.1	0.073	163	0.105	...	....
Dec.												
1	174	0.116	5.1	0.064	155	0.089	7.5	0.036	173	0.088	7.8	0.031
2	152	0.118	5.8	0.033	132	0.121	6.0	0.060	182	0.099	9.0	0.029
3	175	0.097	8.2	0.034	150	0.121	6.0	0.053	176	0.089	7.3	0.029
4	135	0.098	3.7	0.069	122	0.142	...	....	145	0.087	6.7	0.025

Dec.	Urine ml/24 hr.	Riboflavin μg/24 hr.	Riboflavin μg/ml	Urine ml/24 hr.	Riboflavin μg/24 hr.	Riboflavin μg/ml	Urine ml/24 hr.	Riboflavin μg/24 hr.	Riboflavin μg/ml
13	1668	170	0.102	983	160	0.163	1032	139	0.135
14	2660	167	0.063	3017	161	0.053	1999	126	0.063
15	1312	174	0.133	1114	156	0.140	1306	137	0.105
16	2494	155	0.062	2917	156	0.053	1985	108	0.054

days for the three subjects. The above authors also state that for the most part the micrograms of riboflavin per milliliter in fasting morning specimens agreed well with the corresponding figures for 23-hour samples. This was true only for the specimens for subject A on November 30th. On December 14th and 16th, when hourly urine samples for 6 hours were desired for ascorbic acid excretion tests (Delaney, '45), it was necessary for subjects to drink more water than on the 13th and 15th. Comparison of the riboflavin excretion values for these 4 days shows much closer agreement between the micrograms per 24 hours than per milliliter. The results of the present study do not support the suggestion that urinary riboflavin excretion is related to urinary volume.

Fecal synthesis and excretion of riboflavin. Values for the average daily fecal excretion of riboflavin are included in table 1. Considerable amounts of "free" riboflavin were found in the feces of all three subjects on the synthetic diet. The amounts on the natural diet were 2.9, 3.7, and 3.0 times greater than those found on the synthetic diet. The "combined" riboflavin (that found within the bacterial cells, and freed for analysis by action of Mylase P) showed even greater increase on the natural diet, undoubtedly due to an increased bacterial content of the feces. The total riboflavin excretions on the natural diet were 2.8 times greater than those on the synthetic diet for all three subjects.

The total amounts of riboflavin excreted by these three subjects on the synthetic diet approximate half the intake, and consequently indicate nothing concerning riboflavin synthesis. On the natural diet, however, the average total excretion of riboflavin for the last 15 days was greater than the intake for subjects A and C, and only 268 μg less than the intake for subject B. The fecal riboflavin for subject B was considerably below that for subjects A and C throughout the study.

In the studies by Najjar et al. ('44), the fecal riboflavin excretions by boys on synthetic diets containing only 70 to 90 μg of riboflavin per day, varied from 80 to 1276 μg, but

the majority of the values were between 200 and 600 μg per day. In the present study the total fecal excretions on the synthetic diet varied between 80 and 571 μg, but were generally between 200 and 500 μg. The fecal excretions were comparable in the two studies.

On natural diets containing from 0.28 to 0.66 mg riboflavin per 1000 cal, or absolute values of about 500 to 1400 μg per day, excretion values varying from 399 to 872 μg per day were found by Davis ('45) in five women subjects. Values for a given individual seemed to be independent of the riboflavin content of the diet. The three subjects on the present study excreted 657 to 1646 μg per day in the feces while they were on the natural diet, with 10 of the 12 values between 840 and 1375 μg. In all of these studies individual differences in fecal riboflavin excretion were noted. Differences in the composition of the diets in these studies appear to be more important in determining fecal riboflavin excretion than are riboflavin intakes.

The evidence presented in this report leads to the conclusion that the conditions when the natural diet was used were more favorable for bacterial synthesis of riboflavin than those when the synthetic diet was ingested. What effect this increased synthesis may have had on the absorption and urinary excretion of riboflavin is not clear, since the riboflavin intakes were higher on the diet producing the larger amount of riboflavin synthesis.

Value of riboflavin excretion studies as a measure of requirement. Studies have been reported from several laboratories in which subjects on very low riboflavin intakes have excreted considerable amounts of riboflavin, and have exhibited none of the expected symptoms of riboflavin deficiency (Najjar and associates, '44; Hagedorn et al., '45; and Keys et al., '45). Work of Michelson, Doeden and Keys ('45) has demonstrated that urinary riboflavin excretion is not related to riboflavin intake only, but may be significantly altered by factors such as complete starvation, hard physical work, acute

athiaminosis, and bed rest. The true significance of urinary excretion of riboflavin has not yet been established, and all factors affecting it are not known. The whole picture is complicated by the fact that the specificity of the formerly accepted symptoms of deficiency, corneal vascularization and cheilosis, has been questioned.

Extensive evidence has been given in papers mentioned above of total excretions of riboflavin far in excess of the intakes over considerable periods of time. The evidence of large amounts of "free" riboflavin in the feces suggests that this might be a source of the urinary riboflavin, but no means of measuring conditions leading to its absorption have been suggested. May there be appreciable absorption of fecal riboflavin when the riboflavin concentration in the body is low, and less absorption at other times? Such a condition has been shown to exist in regard to iron (Ross and Chapin, '41). Evidence has been presented (Najjar et al., '44) that riboflavin is not excreted into the intestinal tract.

Excretion studies add to our information concerning riboflavin metabolism, but do not give us the answer to the question of requirement.

SUMMARY AND CONCLUSIONS

Three normal women were maintained for 7 weeks on a synthetic diet containing 1.09 mg of riboflavin per day. This diet was followed, after a month's respite, by a diet of natural foods containing 1.33 mg riboflavin per day. Throughout the study 24-hour urinary excretions of riboflavin were determined, and during 2 weeks of the time on the synthetic diet, 1-hour fasting excretions were also measured. Fecal riboflavin excretions were measured by 5-day periods. The results may be summarized as follows:

1. Daily urinary riboflavin values for the three subjects averaged 165, 152, and 161 μg on the synthetic diet, and 174, 229, and 210 μg on the diet of natural foods.

2. The 1-hour fasting urinary excretions of riboflavin varied from 3.7 to 10.9 μg, with average values for the three subjects of 5.9, 7.1 and 7.8 μg.

3. Urinary riboflavin excretion showed no relation to urinary volume.

4. Fecal riboflavin excretions on the diet of natural foods were 3.7 to 3.8 times greater than those obtained on the synthetic diet.

5. On the diet of natural foods, total riboflavin excretions were 2.8 times greater than on the synthetic diet for all three subjects, and for two subjects exceeded the intake.

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THE DEVELOPMENT AND DEMONSTRATION OF CORNEAL VASCULARIZATION IN RATS DEFICIENT IN VITAMIN A AND IN RIBOFLAVIN

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TWO PLATES (TWELVE FIGURES)

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The invasion of the cornea by capillaries has often been considered to be a symptom of nutritional deficiency disease. This vascularization of the cornea has been observed in rats deficient in riboflavin (Bessey and Wolbach, '39), vitamin A (Wolbach and Howe, '25), tryptophane, lysine, zinc or sodium (see the review of Dann and Darby, '45). We have recently reported a similar invasion to take place in rats subjected to a methionine-deficient diet or to a diet devoid of protein (Sydenstricker, Hall, Hock and Pund, '46). In man corneal vascularization has been observed to result from riboflavin deficiency (Sydenstricker, Sebrell, Cleckley and Kruse, '40). This responds to riboflavin therapy (Sydenstricker, Kelly and Weaver, '41). In both rats (György, '42) and man (Lyle, Macrae and Gardiner, '44) there seem to be unknown nutritional factors of importance in preventing vascularization of the cornea. It is of interest that thallium poisoning (see Buschke, '43) or a high tyrosine diet (Hueper and Martin, '43) can result in vascularization of the cornea.

Physical and chemical trauma and infections may also result in corneal vascularization (Duke-Elder, '44). Lowry and Bessey ('45) have recently investigated the effects of light, trauma, riboflavin and ariboflavinosis on the production of corneal vascularity. "Brilliant continuous illumination with incandescent lamps did not augment the changes in the cornea and conjunctiva resulting from riboflavin deficiency" nor did the illumination affect the riboflavin content of the cornea (Bessey and Lowry, '44). Corneal changes in ariboflavinosis appeared when the riboflavin content of the cornea had fallen to less than half of normal. This would seem to indicate that the corneal changes were evidence of rather extreme depletion of riboflavin. The administration of extra riboflavin to normal rats had no detectable influence on the development of "spontaneous" corneal vascularity (Lowry and Bessey, '45). The administration of riboflavin has also been shown to have no effect on the corneal vascularization resulting from tryptophane deficiency (Albanese, '45) or that resulting from deprivation of protein (Sydenstricker, Hall, Hock and Pund, '46).

All in all, the mechanism of corneal vascularization, as well as some of the causes of this condition, seem to be obscure.

We have undertaken to investigate possible causes for the development of corneal vascularization and, at the same time, to try to accumulate information which might lead to a better understanding of this phenomenon. The present paper is concerned with a description of the methods and techniques which we have adopted, with the variation seen in the normal rat cornea, and with our observations on the corneal changes resulting from riboflavin and vitamin A deficiencies.

METHODS

In our investigation of the normal variation in the cornea of the rat with the biomicroscope, we have examined the eyes of some 500 of our Wistar strain rats. All of these rats up to the time of examination were on a diet of commercial dog food.[1] In

[1] Purina Dog Chow Checkers.

addition, they received milk daily and ample amounts of lettuce and carrots at least three times a week. Of the 500 rats used, 78 were adult rats when first examined and 20 others were followed from weaning to adulthood with periodic biomicroscopic examination. Two hundred and sixty-five rats were from 21 to 40 days of age when examined and the remainder of the rats were of ages varying from 40 to 120 days.

Nine rats from three litters and later two litters of seven and of six rats, respectively, were placed on a riboflavin-deficient diet when from 25 to 29 days of age. Eight rats from the first three litters and twelve other rats from different litters of the same strain were placed on the control diet. The experiment was further controlled by producing regression of corneal changes with the administration of riboflavin to some of the rats. The riboflavin-deficient diet consisted of vitamin free casein [2] 20 gm, salt mixture (as used by McKibben, Madden, Black and Elvehjem, '39) 4 gm, cod liver oil (U.S.P.) 2 gm, cottonseed oil 2 gm, sucrose 71.7 gm, choline chloride 250 mg, thiamine 10 mg, pyridoxine 10 mg and calcium pantothenate 20 mg. The last mentioned group of six rats on the riboflavin-deficient diet received a supplement of small amounts of riboflavin as follows: At the end of the second week on the diet, a supplement to supply 3 μg of riboflavin per day was started. This was administered in a 20% solution of ethyl alcohol, twice a week. Since some of the animals had not developed corneal vessels, 50 days later this was cut to 2.4 μg per day. The control diet was similar to the riboflavin-deficient diet except that it contained 1.6 mg of riboflavin per 100 gm of diet.

In order to follow the corneal changes resulting from vitamin A deficiency, a litter of six Wistar strain rats was placed on the vitamin A deficient diet when 25 days of age. Later, another litter of six was placed on the same diet when 41 days of age and a third litter of five at 36 days of age. The diet which they received consisted of vitamin free casein [2] 20 gm, salt mixture (as used by McKibben, Madden, Black and

[2] Labco.

Elvehjem, '39) 4 gm, sucrose 73.8 gm, cottonseed oil 2 gm, choline 200 mg, calcium pantothenate 2 mg, riboflavin 1.6 mg, thiamine 4 mg and pyridoxine 4 mg. An equal number of control rats of the same strain, but of different litters, were placed on the same diet as above, except that 2 gm of the sucrose had been replaced with cod liver oil, U.S.P.

All the rats on deficient diets were kept in wire cages with screen bottoms, one or two rats to a cage, with food and water supplied ad libitum. The rats were weighed twice a week.

Animals with typical corneal changes for histological studies or injection and subsequent photographing of the cornea were selected with the use of a Bausch and Lomb "Universal slit lamp." For biomicroscopic examination of rat eyes, the slit lamp assembly was equipped with a viewing platform similar to that pictured by Buschke ('43). Berliner ('43) has described the technique for the use of the biomicroscope, which we have used in looking for corneal changes. The eyes of all rats on deficient diets were examined with the biomicroscope at least once a week and usually, during periods when eye changes were occurring, examinations were made daily or bidaily. In describing corneal changes we limit our use of the term "corneal vascularization" to the situation where vessels have penetrated the cornea beyond the limits of variation found in the normal rat.

In order to demonstrate the vascular patterns in corneal vascularization a method similar to that of Bessey and Wolbach ('39) has been used here (Cochran, DeVaughn and Allen, '42) where, after India ink injection of the vessels, a photomicrograph was made of a flat mount of the cornea. For our present studies we have developed a method for photographing the intact cornea which is somewhat like the technique mentioned by Eckardt and Johnson ('39). A description of the technique we use follows:

In preparation for making photographs of the intact cornea, the rats were first killed by the inhalation of chloroform. The anterior chest wall was then cut away and the internal mammary arteries and the descending aorta were clamped with haemostats. A 20-gauge

hypodermic needle (the point of which had been partly ground off), was passed through the left ventricle and into the ascending aorta where it was secured with a silk ligature. Approximately 5 ml of Higgin's India ink was injected slowly, after which the head was removed and fixed in 1 : 10 formalin.

After sufficient time had elapsed for thorough fixation, the eye was removed by gross dissection. Then, under a binocular dissecting microscope, the conjunctiva was trimmed at the line of reflection at the fornix and all connective tissue and muscle attachments posterior to this line were dissected off. The posterior portion of the eye was next removed by making a circular incision through the sclera and retina just posterior to the ciliary body. This was followed by the removal of the lens. The ciliary body with the iris attached was freed from its attachment to the sclera and removed. The remaining specimen thus consisted of the cornea, the sclero-corneal junction and a narrow rim of sclera. In order to prevent drying, all of the dissection was done with the specimen partially immersed.

The specimen was photographed with the use of a Leitz microscope and a Makam photomicrographic camera (1 ×). A no. 2 photoflood bulb was used for direct substage illumination, with the top lens removed from the Abbé condensor. A Micro Summar photo-objective with focal length of 40 mm was used at f. 24 with a tube length of 120 mm and 6 × eyepiece. An adapter was devised which made it possible to use Eastman lantern slide plates (medium) in the camera.

In general, for the investigation of histological changes in the cornea, standard techniques were employed which need not be described here. A description of the histological changes in the rat corneas which resulted from the nutritional deficiencies will be described in a later paper.

RESULTS AND DISCUSSION

The cornea of the normal young rat is characteristically clear and limpid. With age there occurs a slight thickening accompanied by some loss of clarity. Also with age there seems to occur a widening of the vascular limbic area and by the time the rat reached adulthood an occasional capillary loop may appear to extend into the edge of the cornea as much as a tenth of the way to the center.

Figures 1 and 2 which are injected corneas from two rats 6 months of age show the degree of vascularity ordinarily

encountered in the limbus of the normal rat's eye. On examination of the limbic area in these two pictures, it is to be noted that there are variations in the width of the limbus due to unequal extension of the vascular transparent scleral margin onto the cornea. These pictures show the approximate limits of normal variation as commonly encountered, so only when corneal capillaries extend beyond the scleral margin as shown here and into the cornea do we apply the term "corneal vascularization." As previously mentioned, the vascular area at the sclero-corneal junction is likely to be narrower in younger rats.

Buschke ('43) states that "recent observations with the slit lamp on a highly inbred strain of rats suggest that vascularization of the cornea occurs also in certain strains of rat as a genotypical trait when the animals are receiving normal diets." Figures 3 and 4 show the opposite sides of a cornea from one of the four animals having apparent spontaneous corneal vascularization, which we have observed among the 500 animals of all ages examined. In these animals the vessels were few and scattered and in marked contrast to the heavy vascularization resulting from most of the types of nutritional deficiencies which produce vascularization, or to the vascularization accompanying an inflammatory reaction. No signs of inflammation or injury to the cornea were seen with the biomicroscope and the vessels were observed to be more or less bilaterally symmetrical. It seems unlikely that the vessels were due to trauma or that they resulted from nutritional deficiency. All four rats in which the vascularization was observed, since weaning had been on a diet of commercial dog food [1] supplemented at least three times a week with milk, lettuce and carrots. Lowry and Bessey ('45) found that this type of corneal vascularization did not regress on the administration of riboflavin. The injected cornea shown in figures 3 and 4, which was from a rat 60 days of age, we consider to be a more or less typical instance of this type of "spontaneous" corneal vascularization.

In riboflavin deficiency the first ocular changes are conjunctival edema and congestion and about a week later marked congestion of the limbic vessels and occasionally faint nebulae in the cornea. Soon thereafter capillary "sprouts" of the terminal loop type may be seen to extend a short distance into the cornea, usually less than ¼ the radius of the cornea.

A few days later intense edema and opacity of the cornea develop so that it is not possible to determine the extent of the vascular invasion. After 7 to 14 days the edema and opacity subside and in the great majority of rats, the cornea is seen to be extensively vascularized, often with capillaries anastomosing across the center. The vascular pattern is apt to be of the terminal loop type although some rats show dendritic patterns. Some rats show cataracts at the time that resolution of corneal opacity occurs; others develop it from 7 to 14 days later. Photophobia is usually obvious from the time that the first ocular changes can be seen.

In about 60% of the rats the first visible invading capillaries seem to stem from the circumferential artery in the superior nasal quadrant; later invasion occurs from the entire complex of circumferential arteries. In about 20% the first invasion is from the "feeder" arteries which rather regularly enter the limbus immediately above the pupil at "12 o'clock" on the circle of the cornea. In about another 20% primary invasion is from the superior temporal quadrant. In general, our observations confirm those of Bessey and Wolbach ('39) with the exception that with frequent biomicroscopic examination of the eyes of the deficient rats, we have observed a transient opacity of the cornea which occurs early in the invasion of the cornea by capillaries. The cause of this opacity will be discussed in a later paper.

At the time of death four of nine in the first group of riboflavin-deficient rats showed minor corneal changes. Two had early corneal vascularization. These rats were 29 days of age at the time they were started on the deficient diet. Of the litter of seven rats which were placed on the riboflavin-deficient diet when 25 days of age, only one showed corneal

changes. This rat at death had numerous vessels extending to a length of more than a fourth of the distance to the center of the cornea. The third group of six rats which were 29 days of age when placed on the riboflavin-deficient diet, all developed extensive corneal vascularization and the typical corneal changes described above. Anterior and oblique views of injected corneas from two of these rats are shown in figures 5, 6, 7 and 8. One rat in the group developed a typical riboflavin cataract. These rats were the ones which received the supplement of small amounts of riboflavin as previously described. None of the control rats showed significant corneal changes on biomicroscopic examination. The riboflavin-deficient diet used would appear to be very low in riboflavin; in fact, the only constituent of the diet containing appreciable amounts of this vitamin is probably the Labco casein. According to Cannon, Boutwell and Elvehjem ('45) samples of this casein preparation were found to contain 0.120 mg of riboflavin per 100 gm of casein. The rats on the riboflavin-deficient diets during the course of the experiment consumed decreasing amounts of the diet starting at about 10 gm of diet per day. This would supply through the casein portion of the diet a maximum of 2.4 μg of riboflavin per rat per day. It appears that in order for the typical corneal changes of riboflavin deficiency to develop, the rats should receive sufficient riboflavin to survive until the corneal changes appear. The rats in the first two groups died before sufficient time had elapsed for the corneal changes to develop, or died just as these changes were first observed.

In vitamin A deficiency as in the ocular manifestations of other deficiency diseases, conjunctival edema and congestion are apt to precede changes in the cornea. Later xerophthalmia develops. The cornea loses its luster, the surface appears slightly opaque and granular and masses of desquamated epithelium from the palpebral conjunctiva may be seen adherent to the cornea. A few days later (3 to 7) there is general thickening and slight diffuse opacity of the cornea and invading capillaries may be seen originating from the same portions

of the circumferential arteries that have been noted in the description of riboflavin deficiency. From the first the invading capillaries in vitamin A deficiency are apt to be of the dendritic type and frequently they tend to form a dense collar of vascularity extending from ½ to ¾ the radius of the cornea. More rarely the invading capillaries are of the terminal loop type and extend entirely across the cornea, anastomosing with loops from the opposite side. After the establishment of a dense corneal plexus, edema and leukocytic infiltration of the cornea are apt to progress to the point where the vessels may no longer be seen with the slit-lamp. As in riboflavin deficiency, evidences of photophobia are commonly present from the time that conjunctival changes are visible. Our observations of the effects of vitamin A deficiency on the cornea of the rat in general confirm the observations of Wolbach and Howe ('25) and of Bessey and Wolbach ('39).

Of the litter of six rats which were placed on the vitamin A-deficient diet when 25 days of age, ocular changes were observed in only one rat. A few days before this rat died, it developed a dense corneal opacity and began to show signs of xerophthalmia. The rats of this litter died on the average in 54 days after being placed on the diet. On a previous occasion we had placed a litter of four rats on a vitamin A-deficient diet when 22 days of age, all of whom died without developing xerophthalmia or corneal vascularization.

Of the litter of six rats placed on the vitamin A-deficient diet at 41 days of age, five showed typical ocular symptoms of vitamin A deficiency as described above beginning about 70 days after being placed on the diet. Figures 9, 10, 11 and 12 are pictures of injected corneas from three of these rats. The cornea shown in figure 9, at the time the animal was killed, showed vascularization only in the superior nasal quadrant, while figures 10 and 11 show the further development of vascularization.

Two rats from the third litter of five which were 36 days of age when placed on the diet, developed early xerophthalmia with a mild degree of corneal vascularization before they died.

The other three rats showed no ocular changes. The rats from this litter lived an average of 78 days after being placed on the diet. None of the rats on the control diet showed any significant eye changes on biomicroscopic examination.

The vitamin A-deficient diet was probably very low in vitamin A. The cottonseed oil used gave no test for carotene with antimony trichloride reagent and probably none of the other constituents contained very appreciable amounts of the vitamin. While it is difficult to draw any very adequate conclusions from four groups of rats, it would seem that the younger rats did not live long enough on the deficient diet to develop the characteristic corneal lesions. Only the rats in the oldest litter which were 41 days of age when placed on the diet, lived to show all the typical corneal changes of vitamin A deficiency. We had observed that with our rats on diets deficient in protein, lysine, or methionine, the age of the rat and the degree of deficiency of the diet were of importance in determining whether corneal vascularization appeared and relatively how soon it developed (Sydenstricker, Hall, Hock and Pund, '46). What we have observed with rats deficient in vitamin A and riboflavin is consistent with this finding.

The ocular changes in the riboflavin and vitamin A deficient rats described in this paper are typical of what we have observed in the examination at various times of a considerable number of rats which were suffering from one or the other of these deficiencies.

On biomicroscopic examination, it was observed that as corneal vascularization began, capillaries usually first invaded the cornea in the superior nasal quadrant. As vascularization progressed, the vessels in this quadrant usually continued to exceed in length the vessels in other quadrants. Occasionally the vessels from two quadrants would exceed in length the vessels from other quadrants. This seems to hold true in all the types of vascularization due to nutritional deficiencies which we have studied. Figure 9 shows the margin of the superior nasal quadrant of the cornea of a vitamin A-deficient rat in which vascularization has proceeded far into the cornea

in this quadrant but has not begun in the other three quadrants. Figures 5 (riboflavin deficiency) and 11 (vitamin A deficiency) show this preponderance in length of the vessels in one quadrant as vascularization proceeds. Figures 10 and 12 (vitamin A deficiency) are pictures of a cornea where the vascularization was greater in two quadrants. Figure 7 (riboflavin deficiency), on the other hand, shows an instance where the development was approximately equal in all quadrants.

In figures 3 and 4 it is to be noted that the capillaries seem to arch over and tend to form loops over a point in about the middle of the picture. On examining photographs of our injected corneal preparations and the preparations themselves, we find that the developing capillaries, as they grow from the circumferential vessel into the cornea, frequently seem to grow from either side of the feeder vessel and to anastomose over the point where the feeder vessel bifurcates into the circumferential vessel. This, in many cases, causes the capillaries to appear to arch over that point.

It would appear that the pattern of vascularization observed when corneal vascularization results from nutritional deficiencies may depend on several factors. The number of capillaries invading the cornea, the distance to which they have penetrated and the extent to which they branch, are probably of importance. It seems that as the capillaries penetrate the cornea their first portions are relatively straight with only fine branches. If the capillaries are numerous and close together, these branches form numerous capillary loops, but if they are fewer in number and farther apart, the dendritic pattern tends to be more apparent. As the vessels converge over the curvature of the cornea, the branches anastomose and the pattern becomes of the capillary loop type. As the vessels grow farther into the cornea the appearance is again dendritic until, by convergence, they once more form capillary loops.

With vitamin A deficiency the numerous capillaries are close together and their branches result in frequent anastomoses forming many capillary loops or a capillary network while in riboflavin deficiency the less numerous capillaries are

farther apart and anastomose less frequently so that at times the dendritic pattern is observed.

SUMMARY

Techniques for preparing and photographing injected corneas of the rat to best demonstrate vascularization are described.

The variation in vascularity at the limbus in rats of various ages has been investigated in some 500 normal rats.

The corneal vascularization resulting from vitamin A and from riboflavin deficiencies has been followed during its development and the type of vascularization which results is described and illustrated.

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PLATE 1

EXPLANATION OF FIGURES

(All 15 × reduced approximately one-third.)

1 and 2 Injected corneas from normal rats.

3 and 4 Opposite sides of an injected cornea showing "spontaneous" corneal vascularization.

5 and 6 An injected cornea from a rat deficient in riboflavin.

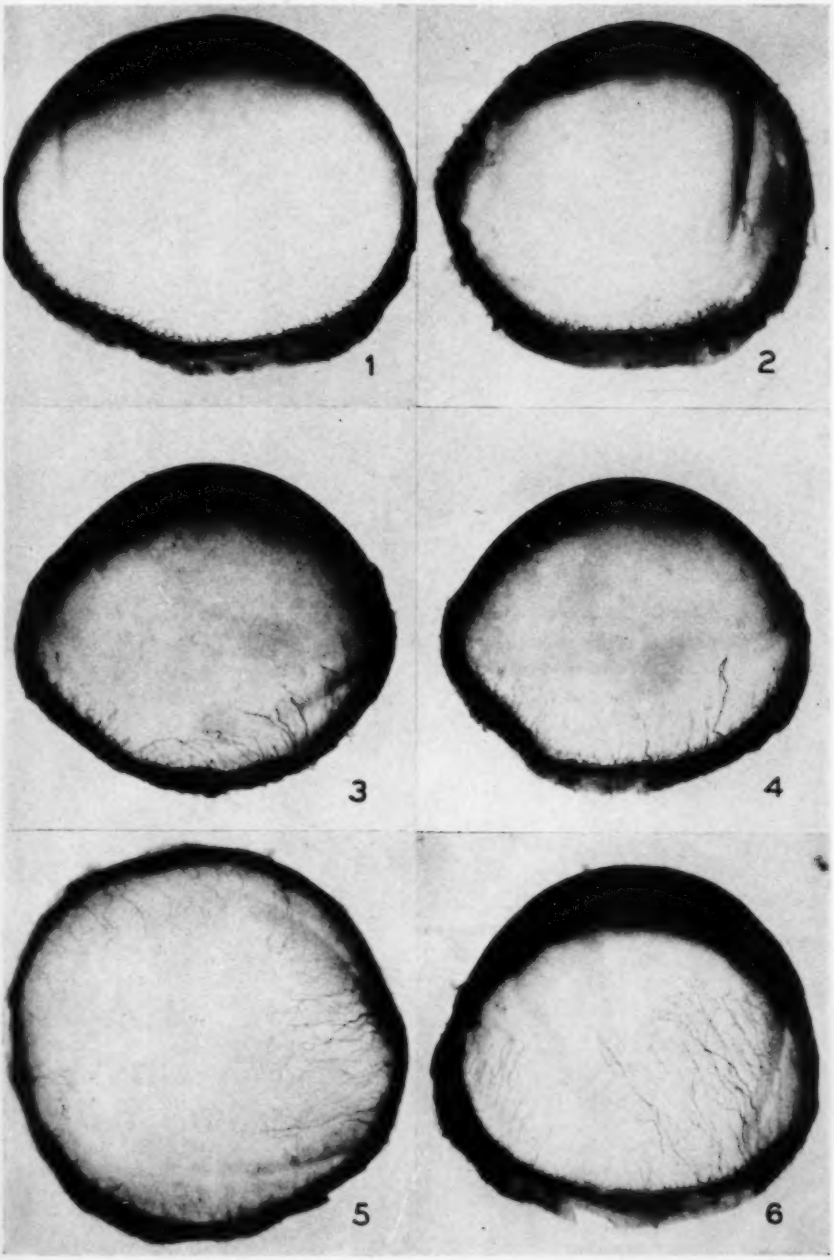
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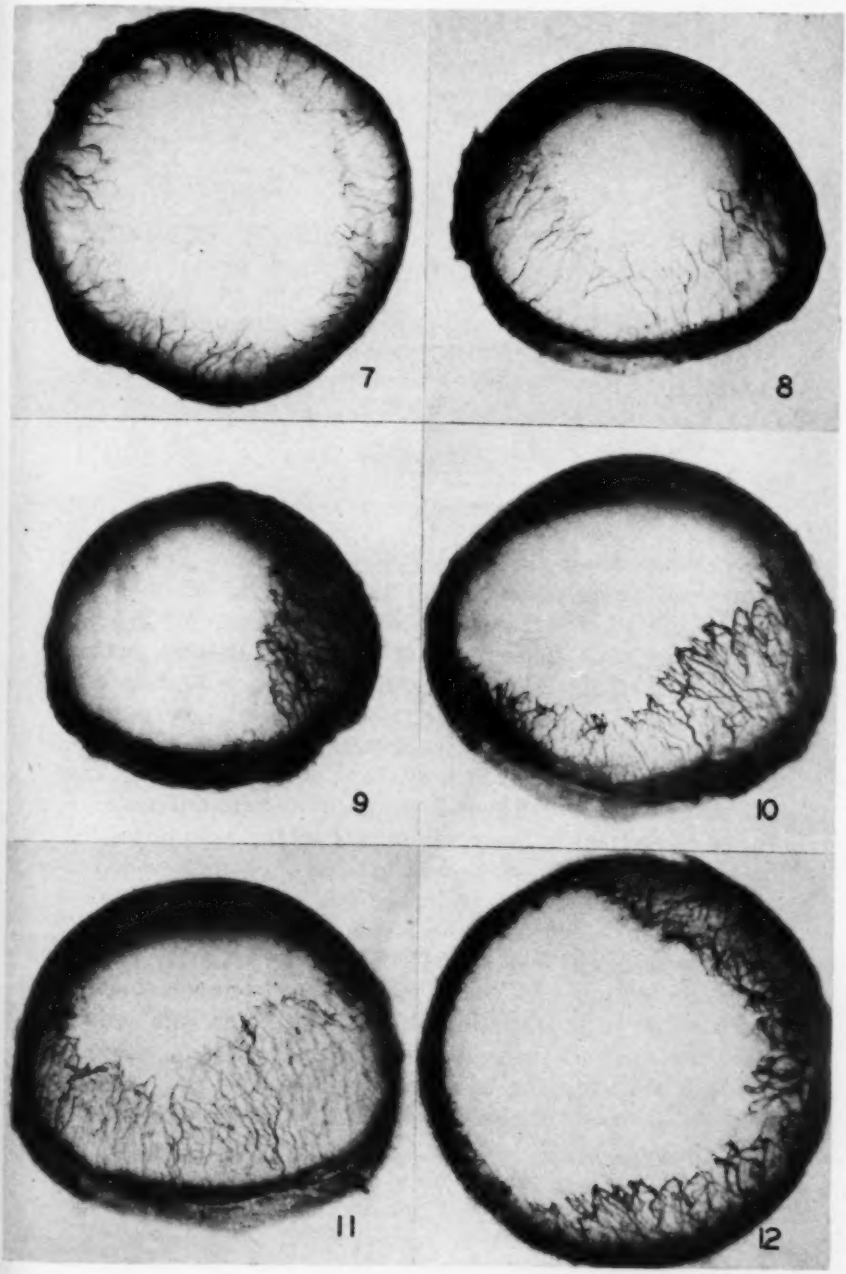
PLATE 2

EXPLANATION OF FIGURES

(All 15 × reduced approximately one-third.)

7 and 8 An injected cornea from a rat deficient in riboflavin.
9, 10 and 11 Injected corneas from three rats deficient in vitamin A.
12 Anterior view of cornea in figure 10.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ATTEMPTS TO PRODUCE A NIACIN DEFICIENCY IN THE RHESUS MONKEY[1]

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THREE FIGURES

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There are comparatively few reports in the literature dealing with the production of niacin deficiency in laboratory animals fed purified rations. Schaefer et al. ('42) demonstrated a niacin deficiency in dogs which were maintained on a niacin-low purified ration. Briggs et al. ('42) reported that the chick requires niacin for optimal growth and for the prevention of chick "blacktongue" when fed a highly purified ration. Wintrobe et al. ('45) in a recent paper on the niacin requirements of the pig reviewed the literature on niacin deficiency in pigs and concluded that most of the results of the earlier workers were equivocal since the natural rations used were low in proteins and probably some of the B vitamins. They (Wintrobe et al.) made an extensive study of niacin deficiency in pigs, and reported that a dietary source of niacin was not required by pigs which were fed a purified diet containing high amounts of protein. Deficiency symptoms did, however, appear when the protein level was low. Wooley and Sebrell ('45) showed that niacin was a dietary essential for rabbits which were fed a purified ration. Although it has been

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shown (Birch, '39) that the rat does not require niacin when fed a purified ration, Krehl, Teply and Elvehjem ('45) found that a deficiency of this vitamin could be produced in the rat when the purified ration was supplemented with corn grits to the extent of 40%. The production of a niacin deficiency in rhesus monkeys has been reported by Harris ('37) and by Chick and Hume ('20) but the rations employed by both these groups of workers were lacking in adequate protein and B vitamins. In this paper we wish to present the results of attempts to produce a niacin deficiency in monkeys using purified rations alone and purified rations with added corn.

EXPERIMENTAL METHODS

The method of handling the monkeys has been described previously (Waisman et al., '43). The basal ration (M-2) consisting of sucrose 73, Labco casein 18, salts IV 4, cod liver oil 3, and corn oil 2 was fed ad libitum. Adequate amounts of ascorbic acid and all the B vitamins (thiamine, riboflavin, pyridoxine, pantothenic acid, choline, i-inositol, p-aminobenzoic acid and biotin) except niacin were fed daily as a separate supplement. Folic acid was also supplied as a niacin low norite eluate concentrate prepared according to the directions of Krehl et al. ('45) at a level equivalent to 5 gm original solubilized liver (fraction L) per day. In later experiments the synthetic Lactobacillus casei factor replaced the norite eluate concentrate and was fed at a level of 100 μg per day. Microbiological assays (Krehl et al., '43) showed that the basal ration together with the supplement contained less than 50 μg of niacin per 100 gm.

Studies with purified rations

Four young monkeys (nos. 186, 187, 188 and 189) were placed on the niacin low purified ration. The monkeys continued to grow at a slow rate for several months without showing any deficiency symptoms. At the end of the fifth month, monkey 188 started to lose weight, and niacin therapy at a level of 10 mg per day was instituted. During the next 4

days on this regimen the monkey continued to lose weight rapidly and 25 mg of niacin amide was administered by intraperitoneal injection. The niacin and niacin amide therapy were completely ineffective and the monkey died the next day. Autopsy revealed no gross lesions.

At the end of the sixth month, monkey 189 showed some weight loss. The hemoglobin values also began to decrease and reached a value of 9 gm per 100 ml of blood. Niacin given at a level of 10 mg per day for 1 week proved ineffective in preventing the weight loss, the decrease in hemoglobin content of the blood and the anorexia. The basal ration was

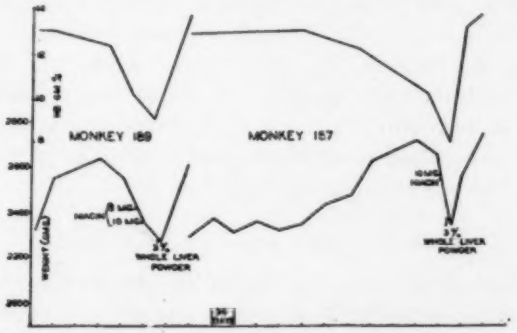

Fig. 1 The response in hemoglobin and weight to niacin and whole liver powder in monkeys which failed on a niacin low purified ration.

supplemented with 3% whole liver powder and fed via stomach tube. Within a week the animal's appetite improved sufficiently to discontinue the tube feeding. Shortly thereafter a sharp increase in weight occurred which was followed by an increase in hemoglobin content of the blood.

Monkey 187 showed a similar history although the animal continued to grow for 14 months before deficiency symptoms appeared. Again niacin was ineffective in correcting the weight loss and drop in hemoglobin, but when the basal ration was supplemented with 3% whole liver powder there was a prompt remission of these symptoms. The data for these animals are summarized in figure 1.

It is apparent from these results that a niacin deficiency can not be readily produced in monkeys fed a purified ration. Since the syndrome precipitated by the niacin low ration was completely reversed by feeding 3% whole liver powder, it is quite probable that a deficiency of the monkey anti-anemia factor existed. These observations are similar in many respects to riboflavin (Cooperman et al., '45b), vitamin B_6 (McCall et al., '46), pantothenic acid (McCall et al., '46), and folic acid (Cooperman et al., '46) deficiencies in the monkey which are complicated by a concomitant deficiency of the monkey anti-anemia factor. There is, however, one distinct difference in the case of niacin, namely, niacin therapy has no beneficial effect upon the deficient animals whereas in the case of riboflavin, vitamin B_6, pantothenic acid, and folic acid there is an initial response to the respective vitamins. The length of time required for the animals to become deficient on the niacin low ration probably depends upon the previous body stores of the monkey anti-anemia factor.

Studies with high corn rations

After having been on the niacin low purified ration for 15 months, monkey 186 still showed no untoward effects. Since Krehl et al. ('45) had demonstrated that rats required niacin when the basal ration was supplemented with 40% corn grits, an attempt was made to produce a niacin deficiency through this means. After 6 weeks on the corn containing ration, monkey 186 began to lose weight rapidly. Twenty-five mg of niacin per day was given for 4 days without any effect on the rate of growth or decrease in hemoglobin. Since Krehl, Teply, Sarma and Elvehjem ('45) had demonstrated that tryptophane could replace niacin as a growth factor for rats when fed the corn grits basal, the monkey was given 300 mg dl-tryptophane per day in addition to the daily supplement of niacin without any beneficial effect. The monkey was then returned to the M-2 basal without the corn grits for 1 week but became progressively worse. The M-2 basal was supplemented with 3 gm of lyophilized liver per day fed via stomach

tube. After 3 days the animal regained its appetite and the tube feeding was discontinued. This treatment was followed by a sharp increase in weight, the animal gained 300 gm in 1 week and the hemoglobin content of the blood increased to 13.5 gm per 100 ml during the same period. The data are summarized in figure 2. The corn grits, in all probability, precipitated a deficiency of the monkey anti-anemia factor since lyophilized liver is an excellent source of this factor (Cooperman, McCall and Elvehjem, '45a).

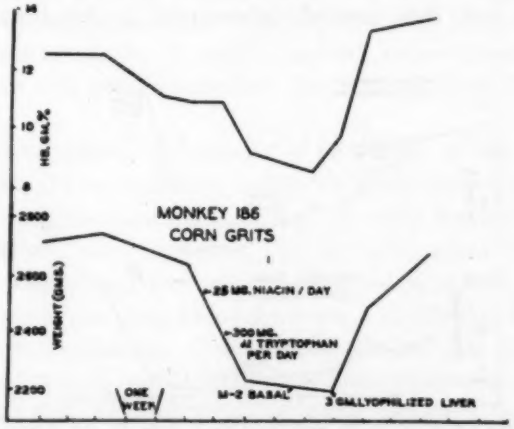

Fig. 2 The effect of feeding niacin, tryptophane, the M-2 basal, and lyophilized liver on the weight and hemoglobin of a monkey which failed on the corn grits basal.

In order to repeat this work with young monkeys, experiments were started in which newly acquired monkeys were placed on the corn grits basal directly. Three young monkeys (nos. 247, 248, and 275) were given the M-2 basal supplemented with 40% corn grits and all the B vitamins except niacin. The hemoglobin content of the blood and differential leucocyte counts were determined regularly on the animals since these are affected in a deficiency of the monkey anti-anemia factor (Cooperman et al., '46; McCall et al., '46). The results with

a typical monkey (no. 248) will be described since all the animals showed a very consistent picture. This animal grew at a very slow rate for 5 weeks at which time growth ceased entirely. The hemoglobin value was 10.6 gm % at this time. The monkey then started to lose weight and at the end of 8 weeks the hemoglobin had decreased to 8.1 gm % and differential leucocyte counts showed a definite reversal in the neutrophile-lymphocyte ratio, the usual syndrome in the monkey anti-anemia factor deficiency (Cooperman et al., '46; McCall et al., '46). At this time 10 mg niacin per day was given for 10 days but this proved inadequate in ameliorating the

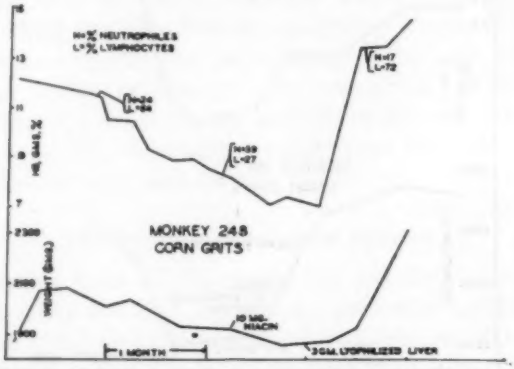

Fig. 3 The effect of niacin and lyophilized liver on the weight, hemoglobin and differential leucocyte counts of a young monkey fed the corn grits basal.

weight loss or the blood dyscrasia. The ration was then supplemented with 3 gm of lyophilized liver per day and within 3 days there was a perceptible change both in the weight and blood picture. At the end of 3 weeks the hemoglobin value reached a level of 14.7 gm % and the neutrophile-lymphocyte reversal was corrected. The data for this animal are summarized in figure 3.

In the case of monkey 247, the corn grits basal was supplemented with both niacin and tryptophane when the weight loss and blood dyscrasia became evident, but this combination also proved ineffective.

Two other young monkeys (nos. 276 and 277) were given the corn grits basal supplemented with 300 mg dl-tryptophane per day and all the B vitamins except niacin. Again growth was poor and the typical blood dyscrasia appeared. Supplementing the ration with 10 mg niacin had no beneficial effect either on the weight or blood condition. One hundred and twenty-five micrograms of crystalline vitamin B_c per day were given to monkey 276 by intramuscular injection for 1 week but this too proved ineffective.

Three monkeys (nos. 295, 296, and 297) were started on the corn grits basal, which was supplemented with all the B vitamins including niacin. A weight plateau appeared at the end of 6 weeks and soon thereafter the typical blood dyscrasia appeared.

Since the typical syndrome of a deficiency of the monkey anti-anemia factor is readily produced when monkeys are fed a 40% corn grits basal, this affords an easy method for the production of assay monkeys. Our previous assay depended upon the weight and hemoglobin response in monkeys which failed to show complete recovery from a riboflavin deficiency after riboflavin therapy (Cooperman, McCall and Elvehjem, '45). Experiments in this laboratory (unpublished data) have shown that both of these types of assay monkeys (corn grits and riboflavin deficient) respond similarly to materials rich in the monkey anti-anemia factor and in no case has any material shown activity in one type without giving a response in the other.

It is interesting to note the different effect the corn grits ration has on the rat and monkey. Whereas in the rat either niacin or tryptophane will correct the deficiency, in the monkey neither tryptophane, niacin nor both are of value in preventing the onset or curing the deficiency.

It will be difficult to ascertain whether the monkey requires niacin until a concentrate of the monkey anti-anemia factor low in niacin is available.

SUMMARY

Monkeys on a purified ration extremely low in niacin develop a deficiency which does not respond to niacin amide therapy. Whole liver powder, a good source of the monkey anti-anemia factor, causes a prompt remission of the deficiency symptoms.

When monkeys are fed a ration containing 40% corn grits, a deficiency characterized by a loss in weight, suboptimal hemoglobin, and a reversed neutrophile-lymphocyte count develops. Neither niacin, tryptophane, nor a combination of the two ameliorate the syndrome. Lyophilized liver or whole liver powder correct the deficiency.

ACKNOWLEDGMENT

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NUTRITIONAL SIGNIFICANCE OF INOSITOL AND BIOTIN FOR THE PIG [1]

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TWO FIGURES

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McRoberts and Hogan ('44) stated that as yet there is no evidence that the pig requires inositol or biotin for growth. Their work, however, indicated that the young growing pig needs some unrecognized vitamins and that these vitamins are contained in water extracts of liver or yeast.

Cunha, Lindley and Ensminger ('46) showed that the pig fed desiccated egg white developed the following syndrome: alopecia, spasticity of the hind legs, cracks in the feet, and a dermatosis of the skin characterized by dryness, roughness, and a brownish exudate. This syndrome was prevented by intramuscular injection of 100 μg of biotin per pig daily.

The experiment which is herein reported was undertaken to determine whether the pig needs inositol and biotin in the ration or whether the pig obtains enough of these two vitamins to satisfy its needs through intestinal vitamin synthesis.

EXPERIMENTAL

Six-week old pigs were used in this experiment. These pigs were farrowed by two Chester White gilts which had been

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[2] This report is from a thesis submitted by D. C. Lindley in partial fulfillment of the requirements for the degree of Master of Science.

bred to a Danish Landrace boar. The dams of these pigs had been fed on a purified ration (sucrose 63.8%, casein 20%, lard 11%, and mineral mix 5.2%) for 52 days prior to parturition and throughout the 6-week lactation period. The purified ration fed to the mothers of these pigs contained only six B-complex vitamins (thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, and choline), and therefore the young pigs were presumably depleted to some degree in biotin and inositol.

The pigs were divided equally as to weight, sex, and age in each lot. The rations fed are shown in tables 1, 2, and 3. The same twelve pigs were used throughout the three phases of this experiment. During the first phase (5-week period) the effect of biotin and inositol on growth of the pig was determined. In the second phase (5-week period), the effect of sulfaguanidine on the biotin and inositol requirements of the pigs was determined. During the third phase (6-week period), the effect of sulfathalidine on the intestinal synthesis of biotin and inositol by the pigs was determined.

The basal ration used in this experiment was the same as that reported previously by Heinemann, Ensminger, Cunha and McCulloch ('46). The ration consisted of casein [3] 26.1%, sucrose 57.7%, lard 11% and mineral mix 5.2%. Water-soluble vitamins were fed (in mg per kg of live weight daily) as follows: thiamine 0.52, riboflavin, 0.12, niacin 1.20, pantothenic acid 0.50, pyridoxine 0.20, and choline chloride 10.00; fat-soluble vitamins were supplied (per pig daily) as follows: vitamin A 5,000 I.U., vitamin D 500 I.U., vitamin E 57 mg, and vitamin K 2 mg.

A modification of the "paired-feeding technique" (Mitchell and Beadles, '30), was used in order to keep the feed intake of all pigs constant. Thus, the feed intake of the pigs was limited to the amount consumed by the pig with the smallest

[3] Casein was prepared by precipitation with HCl and numerous washings with HCl. Casein contained 0.005 μg of biotin per gram. Biotin determinations on casein were made by Miss M. A. McGregor, Assistant Chemist, Division of Home Economics, according to the method of Shull et al. ('42) as revised by Shull and Peterson ('44).

appetite — with the vitamins and sulfonamides studied being the only variables among the lots of pigs.

The pigs were fed in individual feeding stalls and were kept on raised floors in order to prevent coprophagy. The raised floors were washed twice daily. The type of raised floors used are shown in figures 1 and 2.

To prevent the development of rancidity and consequently the destruction of certain vitamins, the purified ration was kept in a refrigerator and not more than 2 or 3 days feed requirements were mixed at any time.

All vitamins were kept in solution in a refrigerator. They were fed to the pigs every other day. The required amount of vitamins fed was measured in a calibrated pipette and placed on a small amount of the feed on the top of the ration at feeding time. In this manner, struggling with the animals was avoided and complete consumption of the vitamins was obtained.

RESULTS AND DISCUSSION

The data in table 1 show that no beneficial effect on growth or efficiency of feed utilization was obtained when either inositol or biotin was fed in addition to the basal ration. No difference in external appearance could be observed between the control pigs or those fed inositol or biotin. It might be of interest to note the very high efficiency of feed utilization made by the pigs in the three lots (table 1). All pigs made a gain of one pound on less than two pounds of feed.

The pigs in all three lots were observed to chew the board fences occasionally. Fence chewing has been observed previously at this station with pigs fed a similar ration containing 6.2% of oat hulls. Therefore, chewing wood may be due to a deficiency of some factor or factors other than a lack of fiber.

Since the addition of biotin or inositol to the basal ration for a 5-week period was of no benefit, it was assumed that the pig is evidently able, through the action of intestinal bacteria, to synthesize enough of these two vitamins to satisfy its needs. In order to determine whether this was true, sulfa

drugs which have been used to inhibit the growth of intestinal bacteria and hence intestinal vitamin synthesis, were used. The data obtained when sulfonamides were fed are shown in tables 2 and 3.

The addition of sulfaguanidine (sulfanilylguanidine), at the levels fed (table 2), had no effect on the inositol and biotin requirements of the pig. No appreciable beneficial result in growth or external appearance was obtained by the addition of inositol or biotin to the ration of the pigs fed the drug

TABLE 1

The effect of biotin (B) and inositol (IN.) on growth.

LOT NUMBER	I	II	III
Ration fed	Basal [1]	Basal + IN [2]	Basal + B [3]
Pig numbers	91, 97, 103, 107	94, 95, 104, 105	92, 93, 101, 106
Initial age, weeks	6	6	6
Number of weeks on trial	5	5	5
Av. initial weight, lbs.	22.4	22.5	22.6
Av. daily gain, lbs.	0.45	0.43	0.41
Av. daily feed consumption, lbs.	0.78	0.78	0.77
Lbs. of feed per lb. of gain	1.73	1.81	1.87

[1] Basal ration contained 6 B-complex vitamins (thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, and choline).

[2] Inositol was fed at level of 100 mg per 100 gm of feed.

[3] Biotin was fed at level of 20 μg per 100 gm of feed.

for a 5-week period. A sub-therapeutic level of the sulfaguanidine was fed during the 5-week experiment. This low level may account for the failure of sulfaguanidine to appreciably inhibit intestinal vitamin synthesis. The drug was not increased to a level greater than 0.75% of the ration toward the latter part of the experiment, since it was thought that sulfaguanidine resistant strains of Escherichia coli might be rather numerous by then. Instead it was thought that using another sulfanamide might be more satisfactory in trying to produce a biotin or inositol deficiency. Since the results obtained with sulfaguanidine were not conclusive in determining whether the pig synthesizes biotin and inositol it was

TABLE 2

The effect of sulfaguanidine (SG) on the response of the pig to biotin (B) and inositol (IN).

LOT NUMBER	IA	IB	IIA	IIB	IIIA	IIIB
Previous ration (table 1)	Basal	Basal	Basal + IN	Basal + IN	Basal + B	Basal + B
Ration fed	Basal	Basal + liver [1]	Basal + SG [2]	Basal + IN + SG [3]	Basal + SG [2]	Basal + B [4] + SG
Pig numbers	91 and 107	97 and 103	95 and 105	94 and 104	93 and 101	92 and 106
Initial age, weeks	11	11	11	11	11	11
Weeks on experiment	5	5	5	5	5	5
Av. initial weight, lbs.	36	36.8	37.3	38	37	37
Av. daily gain, lbs.	0.73	0.76	0.71	0.72	0.70	0.79
Av. daily feed consumption, lbs.	1.35	1.35	1.35	1.35	1.35	1.35
Lbs. of feed per lb. of gain	1.85	1.76	1.90	1.83	1.92	1.71

[1] 1–20 liver powder (Wilson) was fed at a level of 2% of total ration.

[2] Sulfaguanidine was fed at a level equivalent to 0.5% of the ration for the first 4 weeks and at a level equivalent to 0.75% of the ration during the fifth week.

[3] Inositol was fed at level of 100 mg per 100 gm of feed.

[4] Biotin was fed at level of 20 μg per 100 gm of feed.

TABLE 3

The effect of sulfathalidine (ST) on the response of the pig to inositol (IN) and biotin (B).

LOT NUMBER	IAA	IBB	IIAA	IIBB	IIIAA	IIIBB
Previous ration (table 2)	Basal	Basal + liver	Basal + SG	Basal + IN + SG	Basal + SG	Basal + B + SG
Ration fed	Basal	Basal + liver [1] + ST [2]	Basal + ST [2]	Basal + IN [3] + ST [2]	Basal + liver + B [4] + ST [2]	Basal + B [4] + ST [2]
Pig numbers	91 and 107	97 and 103	95 and 105	94 and 104	93 and 101	92 and 106
Initial age, weeks	16	16	16	16	16	16
Weeks on trial	6	6	6	6	6	6
Av. initial weight, lbs.	61.5	63.5	62	63.8	61.5	64.5
Av. daily gain, lbs.	0.89	0.96	0.51	0.98	1.04	0.80
Av. daily feed consumption, lbs.	2.21	2.21	2.17	2.21	2.21	2.21
Lbs. of feed per lb. of gain	2.49	2.30	4.24	2.25	2.13	2.77

[1] Liver ("1–20", Wilson) was fed at a level replacing 2.0% of the ration.
[2] Sulfathalidine was fed at a level replacing 0.5% of the ration.
[3] Inositol was fed at level of 100 mg per 100 gm of feed.
[4] Biotin was fed at level of 20 μg per 100 gm of feed.

decided to try another sulfonamide. Sulfathalidine (phthalylsulfathiazole) was decided upon since it had been used successfully by Miller ('45) as an inhibitor of intestinal vitamin synthesis with the rat.

The data obtained when sulfathalidine was added to the basal ration are shown in table 3. Sulfathalidine, at the level fed, proved to be very effective in decreasing intestinal synthesis of vitamins. The addition of sulfathalidine to the basal ration for 6 weeks caused approximately a 40% decrease in daily gains. In addition, the following deficiency symptoms were observed in lot IIAA where sulfathalidine was added to the basal ration. During the third week that sulfathalidine was fed, pig 95 of lot IIAA began to show a hair loss and the appearance of a dark brown exudate on the surface of the skin. The hair loss was first observed on the posterior part of the ham. The hair loss then progressed over the rump, loin, and sides. At the end of the 6-week period, the pig was largely denuded except for a small amount of hair around the head and neck and some over the back. The greatest area of alopecia over the back was down the midline. Figure 1 shows the hair loss exhibited by pig 95 at the end of the experiment. Pig 95 also exhibited a spasticity of the hind legs. By observation, it appeared that it was painful for the pig to stand on its hind legs. Figure 2 shows the cracking of the feet which occurred in pig 95. The feet were sore, rough, cracked and occasional bleeding was observed. The cracking of the feet was observed at about the same time the hair loss began and the feet became progressively worse during the remainder of the feeding period. Since the pigs were kept on a wire-mesh floor, this may have aggravated the condition of the cracked feet. However, the pigs receiving the same ration plus biotin showed no evidence of cracked feet. There seemed to be a correlation between the amount of hair loss and the severity of cracked feet. The syndrome exhibited by pig 95 was also shown by the other pig (105) fed the same ration in lot IIAA. The syndrome in pig 105 did not develop as rapidly or reach the severity of that in pig 95. Both pigs showed erratic appe-

tites which limited the feed consumption of the other pigs. Both pigs exhibited a hypersensitivity when forced to move.

The addition of biotin to the basal ration plus sulfathalidine (lot IIIBB) prevented the syndrome described for pig 95 fed the same ration minus biotin, and also increased growth by

Fig. 1 Left, pig 95 fed basal ration + sulfathalidine (lot IIAA). Right, pig 106 fed same ration + biotin (lot IIIBB). The syndrome of pig on left was prevented by biotin as is evidenced by normal appearing pig on right. Note spasticity of hind legs, hair loss, dermatosis of skin characterized by dryness, roughness, and a brownish exudate.

Fig. 2 Pig 95 (lot IIAA). Note cracked feet. The feet were sore and bled at times. This condition was prevented by biotin.

57% as is shown in table 3. The syndrome produced above by adding sulfathalidine to the ration and prevented by biotin is very similar to the syndrome produced by feeding desiccated egg white in the ration of the pig (Cunha et al., '46). The syndrome produced by Cunha et al. ('46) was prevented by

intramuscular injection of biotin. This might indicate that the effect of biotin in preventing the syndrome described above was direct and not by stimulating the intestinal synthesis of other factors.

Of much interest is the finding that the addition of inositol to the pigs fed the basal ration plus sulfathalidine in lot IIBB alleviated to a large extent the deficiency symptoms which were prevented by biotin in lot IIIBB. The inositol alleviated the degree of hair loss and the severity of cracked feet which were very marked when biotin was not included in the ration. This tends to show that inositol alleviated biotin deficiency symptoms. A possible explanation for this, is that inositol stimulated certain organisms in the intestinal tract to synthesize biotin.

When sulfathalidine was added to the basal ration, the magnitude of the daily gains were decreased in the following order by the supplements fed (table 3): (1) 1–20 liver powder plus biotin; (2) inositol; (3) 1–20 liver powder, and (4) biotin. Inositol stimulated growth more than biotin, although biotin was more effective in preventing the syndrome caused by adding sulfathalidine to the ration. The fact that the addition of 1–20 liver powder (lot IB — table 2) to the basal ration did not stimulate growth might indicate that the liver, at the level fed and under the conditions of this experiment, did not supply additional factors at an adequate level for stimulating growth. However, this does not mean that the pig does not need unknown factors. Work of McRoberts and Hogan ('44) has shown that the very young pig needs unidentified factors for growth. The work of Cunha, Ross, Phillips and Bohstedt ('44) indicated that an unidentified factor or factors is needed by the sow for reproduction and lactation.

Hegsted et al. ('40, '42) reported that a deficiency of biotin with the chick resulted in hemorrhagic cracks and incrustations in the bottom of the feet. In many cases, the toes became necrotic and sloughed off. Biotin prevented a cracking of the feet in the pig in the trial reported herein as well as in the experiment when desiccated egg white was fed in the ration

(Cunha et al., '46). This would tend to show that the pig is somewhat similar to the chick in this respect.

The efficiency of feed utilization by the pig during the first 6 weeks of this experiment when no sulfonamide was fed (table 1), was very high. The efficiency of feed utilization decreased as the length of time that the pigs were fed the basal ration increased. When sulfathalidine was added to the basal ration, the efficiency of feed utilization was decreased greatly. It took approximately 85% more feed to produce 1 pound of gain by pigs fed the basal ration plus sulfathalidine as compared to those pigs fed the basal ration alone (table 3).

The hair loss observed with these pigs and with those fed desiccated egg white (Cunha et al., '46) would indicate some similarity to the rat in this respect. A hair loss was first observed with the rat, on what was later shown to be a biotin deficiency, by Boas ('27).

Nielsen and Elvehjem ('42) reported that crystalline biotin could prevent or cure the spasticity which develops in rats fed a biotin deficient ration. Smith ('45) reported that a progressive paralysis in dogs was alleviated by biotin in relatively small therapeutic doses and in a comparatively short time. The pigs in this experiment, as well as those fed desiccated egg white (Cunha et al., '46), developed a spasticity of the hind legs which was prevented by biotin.

In a summary of the mode of action of the B-complex vitamins, Harris and Kodicek ('45) stated that inositol may be a "secondary accessory" (i.e., it is not needed directly by animals, but acts mainly in stimulating microorganisms to synthesize other vitamins which are needed.) They recognized, however, that inositol in addition appears to have lipotropic properties for the rat. Pavcek and Baum ('41) were able to cure "spectacled eye" in the rat by addition of inositol to their purified diet. However, Nielsen and Elvehjem ('41) were able to cure a similar "spectacled eye" condition in the rat with biotin. The fact that both groups of investigators were able to cure the "spectacled eye" condition with two different vitamins might well indicate that therapy with one vitamin in

one case may have stimulated the intestinal synthesis of the other. This would mean that the spectacled eye condition is most likely due to a biotin deficiency but that in the work of Pavcek and Baum ('41) inositol may have acted indirectly in stimulating the intestinal synthesis of biotin. A similar postulation might then be made in this experiment, by stating that inositol alleviated the syndrome in the pig prevented by biotin indirectly by stimulating the intestinal synthesis of biotin.

SUMMARY AND CONCLUSIONS

During a 5-week trial, no beneficial effect on growth or efficiency of feed utilization was obtained when either inositol or biotin was added to the basal ration which contained the six B-complex vitamins — thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, and choline. The addition of sulfaguanidine, at the levels fed, had no appreciable effect on the inositol and biotin requirements of the pig.

The following syndrome developed in the pig when sulfathalidine was added to the basal ration: spasticity of hind legs, hair loss, dermatosis of the skin characterized by dryness, roughness, and a brownish exudate, exudate around eyes, and cracks in feet. The syndrome was prevented when biotin was added to the basal ration. This syndrome is similar to that produced with the pig fed desiccated egg white and prevented by intramuscular injection of biotin.

The addition of inositol to the basal ration plus sulfathalidine alleviated to a large extent the deficiency symptoms prevented by biotin. A possible explanation is that inositol acted indirectly by stimulating intestinal synthesis of biotin.

Biotin and inositol are concerned with the efficiency of feed utilization, since a lack of these two vitamins, when sulfathalidine was fed, resulted in smaller gains being obtained when the same amount of feed was eaten by the pigs.

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COBALT METABOLISM STUDIES

II. PARTITION OF RADIOACTIVE COBALT BY A RUMEN FISTULA COW [1]

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Little is known concerning the role of cobalt in animal nutrition; however, the contention that cobalt is essential in the ruminant diet is supported by the increasing numbers of field observations reporting widespread deficiency areas (Beeson, '45). The situation with regard to the essentiality of cobalt in the nutrition of non-ruminants is less clear. Although the extremely small requirement of the animal for cobalt has minimized the opportunities of obtaining the needed information with the ordinary methods of chemical analysis, the use of radioactive isotopes has provided a method by means of which physiological amounts of the element can be employed and traced. The literature on cobalt in animal nutrition has been comprehensively reviewed by Russell ('44).

Emphasis has been placed on experiments with cattle, first because it was logical to use a species for which cobalt is known to be essential and secondly, because of the economic importance of cobalt deficiency diseases in the livestock industry. This paper reports observations on the fate of labeled cobalt administered to a rumen fistula cow, and confirmatory data obtained from range cattle by the slaughter method.

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EXPERIMENTAL

The radioactive cobalt was supplied by the Massachusetts Institute of Technology-Radioactivity Center and was prepared by bombardment of iron with deuterons. It consisted of a mixture of three cobalt isotopes ranging from 65 to 270 days half life and contained 5.45 mg cobalt as cobalt chloride with an initial specific activity of 3303 μg radium gamma-ray equivalent per mg.

The jugular injection experiment was performed about 1 month after the preparation of the radioactive material, while the rumen administration part of the study was carried out after an interval of about 4 months; in the first case the measurements were sensitive to 0.0006 μg cobalt while in the latter work the sensitivity was 0.002 μg cobalt. No inert cobalt was used in preparing the doses. The detection procedure consisted essentially of ashing the sample, bringing the ash into solution and quantitatively electroplating the cobalt onto a copper disk which was used for the actual measurement with Geiger-Mueller counter apparatus. The details of this method have been described by Comar, Davis and Taylor ('46).

The rumen fistula cow, belonging to the Michigan State College herd, was a 1000 lb. Holstein in good health and general condition. It was born on August 28, 1941, and calved the last time on February 24, 1945. A 24-hour collection gave 11.0 kg urine and 22.9 kg feces. The rumen contents, removed 1 day at the usual sampling time, weighed 161 lbs. The blood volume was 27.6 l as determined with Vital red. Milk production during the injection experiment was about 18 lbs. a day; however, it had fallen to about 5 lbs. a day during the rumen administration study. The animal was on a ration that consisted of 15 lbs. alfalfa, 20 lbs. corn silage, 6 lbs. corn and 50 gm salt.

The size of samples used and the preservatives were as follows: 20–40 gm blood, potassium oxalate; 40–80 gm rumen contents, formaldehyde; 40–80 gm feces, concentrated HCl; 30–120 gm urine, toluene; about 300 gm milk, formaldehyde;

and 200–300 gm saliva, formaldehyde. As a rule the larger size samples were used in cases where there was little or no activity. The radioactive cobalt solutions were prepared in Gainesville and shipped to East Lansing for administration to the animal. The preserved samples were returned to Gainesville for the analyses.

On July 20, 1945, at 7:45 A.M. 10 ml of a solution containing 174 μg labeled cobalt as cobalt chloride were injected into the jugular vein of the animal. A blood sample from the opposite side was taken 5 minutes later and the samples then collected periodically, before feeding whenever possible.

On October 11, 1945, at 7:45 A.M. 2 ml of a solution containing 174 μg labeled cobalt as cobalt chloride were introduced, by capsule, directly into the rumen and the samples collected periodically. Since the behavior of cobalt in the rumen was of most interest, it was felt that direct rumen administration was preferable to avoid any possibility of the capsule by-passing the rumen, as might occur after oral administration.

RESULTS AND DISCUSSION

Table 1 presents the data on the fate of the administered labeled cobalt. In each case a complete set of samples was taken before the dosage, none of which showed any activity. This indicated, as expected, that no naturally occurring or extraneous radioactive materials were involved, but of more importance, that there was no residual activity at the time of the rumen administration due to the previous injection.

It is interesting to note the rapid disappearance of the injected cobalt from the blood stream; after 5 minutes only about 6% of the dose was present in the blood of the animal. There appeared to be a certain periodicity, with the blood value falling to 0 at 54, 175 and 319 hours after injection. When the cobalt was administered into the rumen, none was detectable in the blood.

None of the injected cobalt was found in the rumen contents. When the cobalt was introduced directly into the rumen about 82% of the dose was found in the rumen con-

TABLE 1

Partition of radioactive cobalt by rumen fistula cow (dosage 174 μg cobalt).

SAMPLE	JUGULAR INJECTION			RUMEN ADMINISTRATION		
	Hours after dosage	μg Cobalt/100 gm fresh weight	Estimated percentage of dose in whole sample[1]	Hours after dosage	μg Cobalt/100 gm fresh weight	Estimated percentage of dose in whole sample[1]
Blood	1/12	0.034	5.6	9	[2]	0
	1	0.048	7.9	24	[2]	0
	8	0.024	4.0	48	[2]	0
	32	0.0037	0.6	72	[2]	0
	54	[2]	0.0	96	[2]	0
	80	0.018	3.0	120	[2]	0
	104	0.018	3.0	144	[2]	0
	128	0.0053	0.9	168	[2]	0
	152	0.0068	1.1	192	[2]	0
	175	[2]	0.0	216	[2]	0
	197	0.014	2.3	240	[2]	0
	217	0.0074	1.2	264	[2]	0
	247	0.0094	1.6	288	[2]	0
	271	0.0056	0.9	312	[2]	0
	295	0.0035	0.6	336	[2]	0
	319	[2]	0.0			
	344	0.0080	1.3			
Rumen contents	1½	[2]	0	3¾	0.19	81.6
	8	[2]	0	9	0.13	56.3
	32	[2]	0	24	0.12	51.2
	54	[2]	0	48	0.049	20.7
	80	[2]	0	72	0.022	9.4
	104	[2]	0	96	0.0096	4.0
	128	[2]	0	120	0.0043	1.8
	152	[2]	0	144	0.0030	1.3
	175	[2]	0	168	0.0010	0.4
	197	[2]	0	192	[2]	0
	217	[2]	0	216	[2]	0
	247	[2]	0	240	[2]	0
	271	[2]	0	264	[2]	0
	295	[2]	0	288	[2]	0
	319	[2]	0	312	[2]	0
	344	[2]	0	336	[2]	0
Feces	1½	[2]	0	8¾	0.014	0.3
	8	0.020	0.4	24	0.42	18.2
	32	0.0089	2.0	48	0.17	39.5
	54	0.0057	0.9	72	0.024	12.8
	80	0.0051	0.8	96	0.021	2.9
	104	0.0064	0.8	120	0.010	2.1
	128	0.0018	0.5	144	0.010	1.3
	152	0.0037	0.4	168	0.003	0.8
	175	0.0035	0.5	192	[3]	0
Feces (*cont.*)	197	0.0010	0.3	216	[2]	0
	217	0.0017	0.1	240	[2]	0
	247	0.0016	0.3	264	[2]	0
	271	0.0012	0.2	288	[2]	0
	295	[2]	0.0	312	[2]	0
	319	0.0013	0.1	336	[2]	0
	344	0.0012	0.2			
Urine	1½	2.4	7.9	3¾	[2]	0
	7½	0.99	27.8	8¾	[2]	0
	32	0.080	34.2	24	[2]	0
	54	0.025	3.1	48	[2]	0
	80	0.073	3.4	72	[2]	0
	104	0.064	4.4	96	[2]	0
	128	0.012	2.4	120	[2]	0
	152	0.017	0.9	144	[2]	0
	175	0.0058	0.7	168	[2]	0
	197	[2]	0.0	192	[2]	0
	217	0.022	0.6	216	[2]	0
	247	0.0054	1.1	240	[2]	0
	271	0.011	0.5	264	[2]	0
	295	0.0089	0.6	288	0.0067	0.5
	319	0.018	0.9	312	[2]	0
	344	0.010	0.9	336	[2]	0
Milk	8¾	0.0007	0.03	9	[2]	
	81	0.0070	0.3	21	[2]	
	249	0.0025	0.1	57	[2]	
				105	[2]	
				249	[2]	
				369	[2]	
Saliva	1	0.0006		9	[2]	
	247	[2]		101	[2]	
				264	[2]	
				344	[2]	

[1] Estimated percentage of dose: in total blood volume of 27.6 l; in total rumen contents of 161 lbs.; based on daily feces excretion of 22.9 kg; based on daily urine excretion of 11.0 kg; based on daily milk production of 18 lbs.

[2] Amount in sample less than 0.0006 μg.

[3] Amount in sample less than 0.002 μg.

tents after 3¾ hours. The rate of disappearance from the rumen is shown, and after 168 hours less than 0.5% of the original dose was present in the rumen contents. These percentage values are based on the single determination of rumen contents at 161 lbs., however, small uncertainties would not change the picture.

Since it was not possible to make 24-hour collections throughout the experiments, the calculated values for the per cent of dose in the total daily excretions must be considered as estimates only. However, they do indicate the order of magnitude and are helpful in the interpretation of the data. Only about 7% of the injected cobalt appeared in the feces; other data have indicated the probability that much of the injected cobalt reached the intestinal tract via the bile. Where the cobalt was introduced into the rumen, large amounts appeared in the feces within 48 hours, with about 65% of the dose being accounted for. The disappearance of the cobalt from the rumen contents coincided with its appearance in the feces, which may be assumed to indicate lack of absorption.

Large amounts of the injected cobalt were rapidly eliminated in the urine. At 1¼ hours after injection the concentration in the urine was very high, and after 32 hours about 65% of the dose had been eliminated by this path. In the measurements on the urine after rumen administration the sensitivity was such that no activity was found even though samples of about 100 gm were used; however, 311 gm of a mixture of the first five samples gave a value of 0.0006 μg per 100 gm fresh weight, which indicated that an extremely small but definite amount of the cobalt was present in the urine.

Small amounts of the injected cobalt were found in the milk. There were not enough milk samples to give a reliable indication as to the proportion of the dose eliminated by this path; however, it would seem that not more than a few per cent, if that much, could be accounted for in the milk. No activity was detectable in the milk samples after the rumen administration; it should be pointed out that although the

sensitivity was lower in these determinations, amounts comparable with those found in the injection milk samples would have been detected.

An extremely small amount of cobalt was found in the saliva 1 hour after injection. It is not surprising that the cobalt carried by the saliva after injection did not show up in the rumen contents when it is considered that the amount in the saliva was small to start with, and that the rumen contents were considerably diluted with food and water. No cobalt was found in the saliva after the rumen administration.

These observations with the rumen fistula cow have been confirmed by data from other experiments in which the animals were sacrificed at varying time intervals after the administration of the labeled cobalt. The values reported in table 2 were obtained with the following range cattle: cow no. 6, about 5 years old, weight 300 lbs.; steer no. 2, about 2 years old, weight 230 lbs.; heifer no. 3, about 1½ years old, weight 195 lbs.; cow no. 7, about 8 years old, weight 525 lbs., and steer no. 1, about 2 years old, weight 250 lbs.

TABLE 2

Partition of radioactive cobalt by range cattle (slaughter method).

ANIMAL	DOSE μG CO	MODE OF ADMINISTRATION	TIME AFTER ADMINISTRATION	PER CENT OF DOSE IN WHOLE SAMPLE		μG COBALT PER 100 GM FRESH WEIGHT	
				Blood	Rumen contents	Large intestine contents	Urine (not excreted)
			hours				
Cow no. 6	260	Injection	2	3.8	0.17	0.0064	2.2
Steer no. 2	1330	Injection	24	3.0	0	0.40	5.3
Heifer no. 3	2400	Injection	264	0.44	0	0.12	1.2
Cow no. 7	218	Oral	14¼	0	63.8	0.90	0.0015
Steer no. 1	1330	Oral	240	0	0	0	

These animals, in poor condition, were brought to Gainesville from an area in Florida considered deficient in some of the essential minerals. They were then fed on a ration consisting of redtop hay harvested from a low cobalt area, corn grown on a low cobalt area, a protein supplement of dried skim milk, and a phosphorus supplement of tri-calcium phos-

phate. Analyses showed that this feed contained less than 0.01 p.p.m. cobalt which is considered below the maintenance level. For about 6 months under these conditions most of these animals remained in poor condition and gave an appearance of emaciation, rough hair coat, and extreme weakness.

Although these range animals were presumably in a condition of cobalt deficiency the results obtained were in good overall agreement with those reported for the rumen fistula cow. About 3 to 4% of the injected cobalt was present in the blood after 24 hours with the value falling to less than 1% after 264 hours. None of the orally administered cobalt was found in the blood; in the case of cow no. 7, 226 gm of blood were analyzed and the measurements were sensitive to 0.0015 μg cobalt. Here again, with the exception of cow no. 6, none of the injected cobalt was detected in the rumen contents. The cobalt in the rumen contents of cow no. 6 probably reached there via the saliva since this animal received no food or water after the injection and its rumen contents weighed only 26 lbs. About 64% of the orally administered dose was present in the rumen contents of cow no. 7 sacrificed after 14½ hours, while none was found after the 240-hour interval (steer no. 1). The values for large intestine contents and urine (not excreted) agree well with the corresponding values for the rumen fistula cow, especially when it is considered that the range animals were consuming less feed and water, and that steer no. 2, heifer no. 3 and steer no. 1 received larger doses.

It is apparent that the cobalt requirement of the ruminant must be small indeed if retention by the animal is any criterion. Comar, Davis and Taylor ('46) have reported that 10 days after the injection and oral administration of 1 to 2 mg cobalt less than 5 and 1%, respectively, were retained by the cattle. McCance and Widdowson ('44) suggest that the element probably acts upon some of the organisms in the rumen and not on the host, a contention which is supported by observations that cobalt was not curative when given to sheep by injection, and that horses do not apparently suffer from

cobalt deficiency diseases. The findings, reported here, that no significant amounts of injected cobalt reach the rumen contents and no significant amounts of rumen ingested cobalt reach the blood, fit into this picture. The data indicate that cobalt supplements should be supplied at least every week if it is desired to maintain the cobalt level in the rumen contents of animals on feed deficient in this element.

Other studies with radioactive cobalt, many of which are in progress, will be required to permit a decision as to whether cobalt is essential for the normal functioning of haematopoietic or other organs.

SUMMARY

1. When 174 μg labeled cobalt were injected into the jugular vein of a rumen fistula cow about 6% of the dose was present in the blood after several hours with the value falling to 1% or less after 10–15 days. None was found in the rumen contents, about 7% appeared in the feces, and about 65% was found in the urine with large amounts being rapidly eliminated by this path. Very small amounts were found in the milk and saliva.

2. When 174 μg labeled cobalt were introduced directly into the rumen none was detected in the blood. About 82% of the dose was present in the rumen contents after 4 hours with the amount decreasing regularly to less than 1% after 7 days. Over 65% was accounted for in the feces and only extremely small amounts in the urine. No cobalt was detected in the milk or saliva.

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STUDIES ON THE COMPARATIVE NUTRITIVE VALUE OF FATS

IX. THE DIGESTIBILITY OF MARGARINE FAT IN HUMAN SUBJECTS [1]

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The first essential for a food to be considered of high nutritive value is that it be relatively completely digested and absorbed. In an extensive series of experiments carried out by the Office of Home Economics of the United States Department of Agriculture on a wide variety of animal and vegetable fats, it was found that most of the seventy odd fats studied were digested to the extent of 95% or better by human subjects (Langworthy, '23). The exceptions to the almost complete digestibility were fats which had melting points of 50°C. or above such as mutton fat (Langworthy and Holmes, '15), oleostearin (Holmes, '19), deer fat (Deuel and Holmes, '22) and some almost completely hydrogenated fats melting from 52 to 60°C. (Deuel and Holmes, '21).

There is practically no experimental evidence on the digestibility of the modern margarines, most of which are composed of such hydrogenated fats as cottonseed, peanut or soybean oil. Holmes ('25) found that three different types of margarine commercially available in 1915 which were prepared from animal fats were highly digestible. This was true for the sample which contained a considerable portion

[1] This work was carried out under a research grant from The Best Foods, Inc.

of oleostearin even though this fat when fed as the sole fat in the diet, had been shown to be digested to the extent of only 80%.

Since practically complete digestibility has previously been reported for partially hydrogenated cottonseed, corn, and peanut oil with melting points under 50°C. (Deuel and Holmes, '21) as well as a comparable series of blended hydrogenated fats (Deuel and Holmes, '22) (prepared from completely hydrogenated oils mixed with sufficient of the untreated oil to bring the mixture to the desired melting point), there is no reason to suppose that the modern margarines would behave differently. The present tests were carried out in 1938 to determine whether such a supposition is correct. Because of the increasing importance of margarine in the present day diet, these results are now being reported.

EXPERIMENTAL

The digestibility experiments were performed using procedures identical with those employed in the studies of the Department of Agriculture referred to earlier. The subjects were students and technicians closely associated with the Department of Biochemistry who were known to be reliable. There were 5 men and 5 women subjects. All were in normal health.

The experiments were carried out over a 3-day period. During this period the subjects ate a blanc mange pudding consisting of skim milk, cornstarch, sugar, salt, caramel and vanilla into which margarine fat or butter fat was incorporated. In addition to the pudding, whole wheat biscuits, oranges and black coffee with sugar were used. Seven tests were made with margarine fat and three with butter fat. The amount of each food eaten was determined and the total intake of the various foodstuffs calculated from these data. The stools from the experimental period were separated by markers taken with the first meal of the experimental diet and with the first meal following the conclusion of the test.

The stools were dried to constant weight at 95°C. and analyses were made on the pulverized samples for lipid, protein and ash. Carbohydrate was estimated by difference. In estimating the digestibility of the fat, a correction for metabolic fat in the feces was made by multiplying the weight of dry feces by 9.89 (Langworthy and Holmes, '15). The margarine fat was separated from a commercial margarine which was identified as being made from domestic vegetable oils and as having a melting point well below body temperature (94–95°F. Wiley). This melting point is about the average of the margarines currently available, practically all of which vary between 91°F. and 98°F. (Wiley). The butter fat was prepared from a butter widely used in this area.

RESULTS

The results of the tests are summarized in table 1.

TABLE 1

Summary table of digestibility of margarine fat and butter fat in normal men and women.

SUBJECT	SEX	WEIGHT	FAT EATEN	DIGESTIBILITY OF THE DIET AS A WHOLE				CORRECTED COEFFICIENT OF DIGESTIBILITY OF FAT
				Protein	Fat	Carbohydrate	Ash	
			Experiments with margarine fat					
		lbs.	*gm*	%	%	%	%	
A.B.	M	185	331.8	82.2	94.9	94.8	64.7	98
H.B.	M	157	218.6	75.0	90.3	96.5	56.2	94
W.B.	F	122	161.2	56.4	91.3	95.8	50.7	96
B.B.	F	121	332.0	78.8	94.2	96.9	70.9	96
L.H.	F	122	215.5	83.1	94.4	95.2	65.0	97
V.H.	M	132	292.5	92.5	97.1	97.9	81.4	99
S.M.	F	112	264.3	83.9	93.1	96.6	72.7	97
Average			259.5	78.8	93.6	96.3	65.9	97
			Experiments with butter fat					
M.G.	F	104	191.0	71.8	96.4	92.7	60.8	100
J.H.	M	136	80.0	50.5	90.1	95.2	30.2	99
L.K.	M	152	167.3	74.1	88.5	97.5	57.7	92
Average			146.1	65.5	91.7	95.1	49.6	97

The average coefficient of digestibility of the seven experiments on margarine is 97 which is identical with the average obtained in the three tests on butter. The average intake of margarine fat was 260 gm for the experimental period with a maximum of 111 gm per day. The average intake of butter was 146 gm for the 3-day period which was somewhat lower than the fat intake in the margarine experiments. The results of the butter tests agree with those reported earlier (97.0) by Langworthy and Holmes ('15). There was no evidence of alteration in the absorption of other components of the diet with either diet. The subjects remained in normal health for the duration of the tests. These data indicate that from the standpoint of digestibility, margarine can be considered as containing a fat of high nutritive value.

SUMMARY

Margarine fat composed of hydrogenated domestic vegetable oils was found to be digested to an average of 97% by normal men and women which was an identical value obtained in three tests on butter fat. There was no evidence of any unpleasant physiological effects when a maximum of 111 gm of margarine fat was ingested daily.

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THE NICOTINIC ACID, BIOTIN, AND PANTOTHENIC ACID CONTENT OF COWS' MILK [1]

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THREE FIGURES

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Milk has long been regarded as one of our most efficient foods, particularly in the case of infants and children where the demands for growth and development are at a peak. While human milk might be considered as the normal and preferred food for the human infant, many babies cannot be, or are not, breast fed. In any case, cow's milk sooner or later becomes one of the most important constituents of the diet of infants and children.

Studies at the Research Laboratory of the Children's Fund of Michigan (Macy et al., '45) are providing much information on the nutrient composition of breast milk under various known or controlled conditions. Some of the corresponding data for cow's milk are already available in the scientific literature. The present study has been planned to add to those data, and designed to parallel the work of the Detroit group with respect to some of the causes of variation in the nutrient composition.

The factors in human milk studied by the Detroit group have included vitamin A and carotene, ascorbic acid, thiamine, riboflavin, nicotinic acid, pantothenic acid, and biotin. With respect to cow's milk, there is considerable information on vitamin A and carotene (Shaw et al., '37; Kramer et al., '38; U. S. Department of Agriculture, '45), ascorbic acid (Kon

[1] This study was aided by a grant from the Nutrition Foundation, Inc.

and Watson, '37; Whitnah and Riddell, '37), thiamine (Houston, Kon and Thompson, '40), and riboflavin (Theophilus and Stamberg, '45; Whitnah, Kunerth and Kramer, '38; Johnson, Maynard and Loosli, '41). Since completion of this work, Pearson and Darnell ('46) have published the results of a study of the thiamine, riboflavin, nicotinic acid, and pantothenic acid in cow's colostrum and milk. Except for isolated assay values, there is no other information available on the nicotinic acid, pantothenic acid, and biotin content of cow's milk. We have attempted to provide such data by a systematic study of the causes of variation of these vitamins in cow's milk.

METHODS OF ASSAY

The assay methods used were basically the same as those published elsewhere, though somewhat modified in details; first, in order to be able to use the same culture media and stock solutions in assays for all three vitamins; and second, to take advantage of apparent improvements suggested in more recent papers.

Our methods were designed for assay of only one type of sample; namely, milk. They are not necessarily applicable to other samples.

Recovery experiments run by adding known amounts of a vitamin to a previously assayed milk sample gave satisfactory results for all three vitamins.

Cultures and inoculum

The organism for assays for all three vitamins was Lactobacillus arabinosus 17–5. The cultures were transferred in the manner described by Krehl, Strong and Elvehjem ('43), but the compositions of the media were somewhat different. It was felt that they were kept in a more vigorous condition by carrying them in an agar medium containing 1% Difco tryptone, 0.1% glucose, 0.2% phosphate, 0.3% calcium carbonate, and an amount of liver extract representing 0.05 pound of liver per liter of medium. For subculturing the organism from one stab culture to another, and in growing inocula, the

broth medium used contained 1% Difco yeast extract, 0.1% glucose, and 0.5% phosphate.

The latter medium could be used in growing inocula in assays for all three vitamins, but its use required washing of the cells. The cells of a 24-hour culture were centrifuged down, resuspended in 10 ml of sterile 0.9% saline solution, and again centrifuged and resuspended. In the nicotinic acid assays, one drop of the latter saline suspension was used to inoculate each tube. In the biotin and pantothenic acid assays, it was diluted ten-fold before use. In these assays, it was found to be essential that the final inoculum be homogeneous. Any large clumps of cells were allowed to settle or were removed by slow centrifugation before diluting an aliquot of the supernatant suspension.

Basal media

The detailed composition of the media used for the assays is given in table 1.

The hydrolyzed casein was prepared by the method of Krehl, Strong and Elvehjem ('43). The inorganic salts solutions A and B were the same as those of Snell and Wright ('44). The alkali-treated peptone was prepared as described by Strong, Feeney and Earle ('41). The yeast extract was a 10% solution in 0.5 N sodium hydroxide, autoclaved for 30 minutes, neutralized and filtered. We found it necessary to avoid using certain samples of Difco yeast extract which were not freed of pantothenic acid activity by this treatment. This was apparently due to the presence of the alkali-stable pantothenic acid-active material reported by Neal and Strong ('43). The rice polish concentrate was a 5% solution (based on the starting material), prepared by suspending the material at pH 1.3 to 1.5, filtering it, and putting the filtrate through a column of charcoal, and neutralizing.

Procedure

The assay tubes were set up in the usual way with graded levels of standard for the standard curve and various levels

of samples. A special syringe with a small bore tip was used to add the 5 ml of basal medium to the 5 ml volume of standard or sample and water. It was thus possible to add it with sufficient force to thoroughly mix the contents of the tube. Glass caps were used on the assay tubes in place of the usual cotton plugs.

TABLE 1

Compositions of the basal media.

COMPONENT	MEDIA FOR	
	Nicotinic acid and biotin	Pantothenic acid
Hydrolyzed casein	10 gm	4 gm
Alkali-treated peptone		10 gm
Alkali-treated yeast extract		2 gm
Charcoal-treated rice polish concentrate		20 ml
l-Cystine	400 mg	200 mg
l-Tryptophane	200 mg	200 mg
l-Asparagine hydrate	500 mg	500 mg
Adenine	20 mg	20 mg
Guanine	20 mg	20 mg
Uracil	20 mg	20 mg
Nicotinic acid	2 mg [1]	
Thiamine	800 μg	
Pyridoxine	1 mg	
Calcium pantothenate	1 mg	
Riboflavin	2 mg	
Biotin	3 μg [2]	
p-Amino benzoic acid	500 μg	
Glucose	40 gm	40 gm
Sodium acetate	40 gm	28 gm
Inorganic salts A	10 ml	10 ml
Inorganic salts B	10 ml	10 ml
Distilled water to make 1 liter [3]		

[1] Omitted in nicotinic acid assays.

[2] Omitted in biotin assays.

[3] The final medium has half this strength, since an equal volume of sample (or standard) plus water is present.

The titration was carried out electrometrically, using a quinhydrone electrode. By using a Ag-AgCl reference electrode in normal KCl, the potential and galvanometer reading fall to zero at a pH of about 7.8. The sharpest end point was found to be obtained at this pH, using the basal medium described previously.

Preparation of samples

Soon after being drawn, the milk samples were refrigerated, and within 24 hours (in nearly all cases) they were frozen in 5 ml portions for storage at — 13 to — 16°C. A small amount of each sample was reserved unfrozen for analysis for fat and total solids by the Mojonnier methods. The stored samples were seldom held for more than a month before being assayed, and experiments showed that the concentrations of nicotinic acid, biotin, and pantothenic acid did not decrease in a much longer period than this.

Assay results with these three vitamins were not increased by treatment with acid or enzymes (polidase, mylase, clarase, or takadiastase plus papain), so that there is apparently no bound nicotinic acid, biotin, or pantothenic acid in milk. Since agreement of results at different levels of the samples was quite satisfactory without any pretreatment other than melting them and diluting them to the proper extent, no other pretreatment was used.

DESCRIPTION OF SAMPLES

In order to isolate the effects of both stage of lactation and season on the vitamin level of the milk, samples were obtained at various stages of lactation from cows freshening at different seasons of the year. For each cow, samples were taken twice daily for the first 2 or 3 days after freshening; on the next 2 days, composite samples were made from the morning and evening milkings; following these, such composite samples were taken at gradually increasing intervals, so that after the first month composites were obtained once a month. When the cow began to dry off, samples were taken more frequently. The total milk yield represented by each sample was recorded. Per cent fat and total solids, as well as the three vitamins, were determined for each sample.

Three cows (nos. 60, 38, and 46) were used from November 3, 1943, when they were approaching the end of their lactation periods, till they dried off near the beginning of the following year. Milk samples from another group of four cows (nos. 16,

68, 7, and 33) were analyzed from the time they freshened in November and early December, 1943, throughout their lactation periods until they dried off in August and September, 1944.[2] The cows of the first group and one other (no. 15) were studied from the time they freshened in March and early April, 1944, till the experiment was terminated at the end of the year, or until an earlier end of lactation was necessary.[3] Two others (Venus and Delle) were followed from the time of freshening at about September 1, 1944, till the last of January, 1945. Cow no. 33 from the second group, freshened again on November 17, 1944, and samples of her milk were analyzed till the end of the year. All of these cows were Holsteins.

Because of difficulties encountered in obtaining satisfactory pantothenic acid assays during the early part of the studies, many of the earlier samples either were not assayed for this vitamin, or the assay results were excluded from consideration.

Since variations in the feed received by the cows appeared to have little effect, in most cases, on the vitamin content of the milk, it seems unnecessary to describe the feeding regime in detail. Cows 16, 68, 33, 46, and 7 were on feeding experiments unconnected with this study during much of their respective lactation periods. Their feeds were varied frequently with respect to hay, corn silage, and the grain mixture. The changes involved particularly the fat content of the rations. All the other cows were fed standard rations according to normal practices of good herd management. The most clear-cut and sustained change undergone by all these cows was their being put on and taken off pasture.

[2] Cow no. 7, however, was still being milked when the experiment was terminated at the end of the year.

[3] Cow no. 60 had a foot infection throughout this lactation period. This kept the milk yield low, and she was finally killed in August, 1944.

Cow no. 38 became sick in September, 1944, and was dried off without further samples being taken.

Cow no. 15 developed mastitis in November, 1944, and was killed. (The proportion of mortalities encountered in this study is not unusual in dairy herds.)

RESULTS

The accuracy usually claimed for microbiological assay methods allows for errors of up to 10%. While the nicotinic acid assay method, as used in this study, was probably much more accurate than this in nearly all cases, the figure of 10% gives a reasonably conservative basis from which to draw conclusions about the levels of each of the vitamins.

Variation between cows

There is considerable variation in the vitamin content of the milk depending on the characteristics of each individual cow. This variability is particularly great in the case of biotin. The average biotin levels of all milk samples during the first 4 months after the colostrum period for each of eight cows were 11, 12, 15, 17, 18, 20, 28, and 37 μg per liter. The disparity among individual cows in this respect was much greater during the colostrum period. These facts are also made evident by the relative large values of standard deviations of the means shown in figure 2, in which the relation of biotin level to stage of lactation is represented. Of all milk samples assayed, the maximum level for biotin was 56 μg per liter and the minimum was 2 μg per liter. The range for colostrum samples was from 0.6 to 91 μg per liter. The figures given by Hodson ('45) also show a very wide range. The variability of pantothenic acid levels of the milks of individual cows was considerable, but not so great as for biotin. The average calcium pantothenate levels for the 4 months following the colostrum period for each of six cows were 2.7, 2.7, 3.0, 3.8, 4.5, and 4.6 μg per ml. Similar averages for nicotinic acid levels from each of ten cows were 0.42, 0.48, 0.54, 0.62, 0.69, 0.71, 0.80, 0.85, 0.96, and 1.02 μg per ml. Attempts to relate these variations to milk yield, age of the cow, caloric value of the milk (calculated from its fat and solids content), leucocyte count of the milk, or the per cent of fat or other solids were unsuccessful.

Day-to-day variations

There were found to be day-to-day variations in the vitamin levels of the milk from any given cow. This was not particularly marked in the case of nicotinic acid. Daily composite milk samples were obtained on 4 consecutive days from one cow and on 9 consecutive days from another, and an average difference in the nicotinic acid level from 1 day to the next of 7% was found for each series. The pantothenic acid variations for the same series of milk samples were larger, being 10%. With biotin, the changes were quite unpredictable. The first 4-day series gave biotin levels of 21, 23, 21, and 22 μg per liter, but the cows whose milk was assayed on 9 consecutive days gave values of 30, 40, 21, 17, 22, 21, 21, 30, and 29 μg per liter on those days. We are aware of no reason for either constancy or change in this respect. This inconsistency was unexpected, and was not considered in planning the experiment. It obviously decreases the value of composite samples from a single day's milkings when taken relatively infrequently.

Seasonal variation

The relation of vitamin level of the milk to the time of year is shown in figure 1. These curves show the average levels in the milks of all cows being studied at the time for which a point is given. Such averages were determined at 20-day intervals. The level on any particular date for a given cow was obtained from the individual curve for that cow, drawn by connecting points representing the samples taken. Colostrum samples and samples taken at the time of drying off were not included in the averages. The number of cows represented by any one point varied, since certain cows were freshening and others were not being milked at various times throughout the study. This factor accounts for some of the irregularities in the curve. For example, the point for March 30 on the nicotinic acid curve was relatively high since between then and the preceding point, a cow with a relatively low level died, while another with a relatively high level freshened. The

trend of the nicotinic acid curve is clear, and is believed to be of real significance. Throughout late winter and early spring the nicotinic acid content of the milk gradually decreased until May. From then on, a rise set in until October. On May 9, the range of values for eight cows was 0.22 to 0.77 μg per ml, and on October 16, the range for five cows was 0.56 to 0.96 μg per ml. These points roughly correspond with the time at which

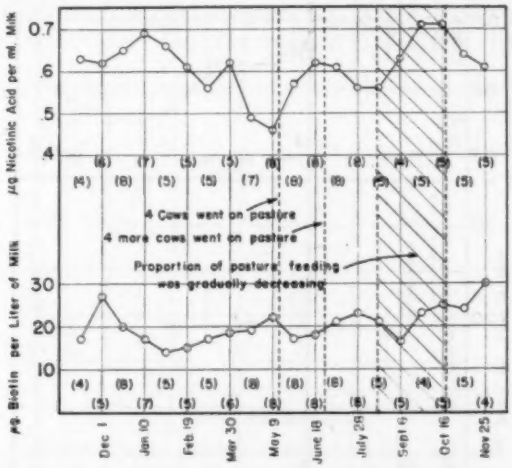

Fig. 1 Seasonal variation of the mean biotin and nicotinic acid levels of cows' milk. The figures in parentheses give the number of cows represented by each point.

the cows first went on pasture and the time of complete cessation of pasture feeding. The irregularities of the biotin curve have no apparent cause. When one considers the variability between cows, the fact that different cows are included in the averages giving the several points, and the day-to-day variation possible for a cow, the irregularities are probably of little significance. The pantothenic acid study was not continued over a long enough period, and differences in stage of lactation at any one date were not sufficient to make any complete analysis of the seasonal variation in pantothenic acid.

Variation with stage of lactation

The relation of the milk, nicotinic acid, and biotin levels to the stage of lactation is shown in figure 2. These curves give the average levels at various intervals throughout the lactation period. They were obtained in the same way as the curves for seasonal variation except that, instead of determining the

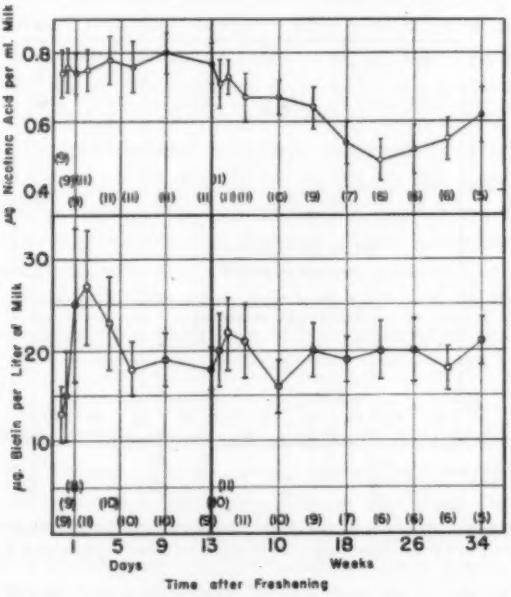

Fig. 2 Variation of mean nicotinic acid and biotin levels of cows' milk with stage of lactation. The standard deviation of the mean is indicated by the spread above and below each point. The figures in parentheses give the number of cows represented by each point.

mean of the levels at corresponding dates from the curves for individual cows, the mean at corresponding points in the lactation period was determined. Again the number of cows represented by the points varies but the variation is much more regular. The number of samples represented by each point becomes smaller as the lactation period progresses because of

the early loss or drying off of some of the cows and the fact that others were not followed through the complete cycle. The standard deviation of the mean has been determined in each case and the values are shown with the curve. They give an indication of the significance of the curve. There appears to be a gradual fall in the nicotinic acid content of the milk throughout the lactation period at least up until the twenty-second week. The extreme variability of the biotin level during the colostrum period is apparent. The initial rise of biotin and subsequent fall to the normal level was quite characteristic

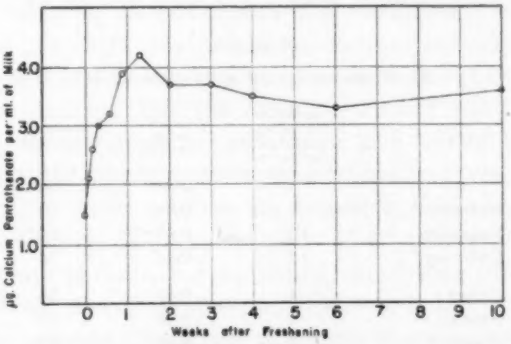

Fig. 3 Variation in mean pantothenic acid level of cows' milk with stage of lactation. Each point up to and including the third week represents seven cows, and the three remaining points represent six cows.

of the curves for the individual cows but the size of the standard deviations of the means makes the evidence for any relation with stage of lactation insufficient. The number of cows represented by the pantothenate curve (fig. 3) was too small to make a calculation of standard deviations worthwhile but the relation here was apparent. The general form of the curve was characteristic of each of the curves for individual cows except for the rise in the level at the end. From an inspection of the individual curves, it appears possible that the latter may be an effect of feed or season. Four cows which went on pasture at approximately this point in their lactation

period showed this secondary rise, while two others which freshened near the first of September did not show such an increase in pantothenic acid at this stage. The first part of the curve agrees very closely with the data of Pearson and Darnell ('46).

Variation with breed of cow

On three occasions a composite sample for each of four breeds was made at milking time using samples from representatives in the University herd. All breeds were therefore being treated alike. The cows involved on each occasion were largely the same individuals. The data appear in table 2.

TABLE 2

Breed comparison of milk vitamin levels.

DATE	BREED	NUMBER OF COWS REPRESENTED IN COMPOSITE	NICOTINIC ACID CONTENT	BIOTIN CONTENT	CALCIUM PANTOTHENATE CONTENT
			μg/ml	*μg/l*	*μg/ml*
2/16/45	Guernsey	8	0.64	19	4.2
3/ 1/45	Guernsey	12	0.50	22	2.2
3/16/45	Guernsey	12	0.65	18	2.6
2/16/45	Jersey	4	0.81	26	5.5
3/ 1/45	Jersey	3	0.76	22	3.3
3/16/45	Jersey	3	0.81	22	3.7
2/16/45	Brown Swiss	9	0.78	29	4.9
3/ 1/45	Brown Swiss	8	0.69	27	2.5
3/16/45	Brown Swiss	7	0.79	24	3.8
3/ 1/45	Holstein	16	0.81	16	2.3
3/16/45	Holstein	14	0.86	14	2.4

We do not consider the data sufficient to establish any significant differences in nicotinic acid content among the Jersey, Brown Swiss, and Holstein milks, but the Guernsey milk appears to be significantly lower. For both biotin and pantothenic acid the order of decreasing vitamin level was Brown Swiss, Jersey, Guernsey, and Holstein. The significance of this order is questionable, however, and the individuality of the cows may well have been more important than their breed.

Relation to other factors

We could not correlate the content of these three vitamins in the milk with other milk constituents determined or calculable, or with other factors. Relations considered were those with fat, total solids, milk-solids-not-fat, approximate caloric value, milk yield, and age of cow. However, there did appear to be some correlation between the pantothenic acid and biotin contents, though the nicotinic acid level seemed unrelated to the levels of the other two vitamins studied. When all milk samples, both normal and abnormal, were considered, the correlation coefficient between biotin and pantothenic acid levels was found to be 0.29. Statistical calculations indicate that the chances are less than one in a hundred that this correlation is fortuitous. Among the six cows mentioned previously in discussing the variation in pantothenic acid level among individual cows, the two highest in pantothenic acid were also the two highest in biotin, and the one lowest in biotin was one of the two lowest in pantothenic acid. If all points [4] including the colostrum periods, but excluding the drying off periods, are considered, the seven cows participating in the pantothenic acid study arrange themselves in exactly the same order with respect to each of the two vitamins. The curves showing the variation of mean vitamin level of milk with stage of lactation may be seen to be of a similar shape for both vitamins. That is, both start from an initially low level of vitamin in the milk, rise to a maximum level (somewhat earlier for biotin), and then fall to a normal level. The observation may be made (for whatever it is worth) that the four breeds studied have the same order, relative to the pantothenic acid and biotin contents of their milks. In none of these respects is nicotinic acid similar to the other two vitamins.

[4] The points were obtained from the graphs for the individual cows at corresponding stages of lactation and mostly at times between those when actual samples were taken. Figure 2 was obtained by averaging such points.

Effect of pasteurization and other treatments

Nicotinic acid, biotin, and pantothenic acid in milk are apparently quite stable. As can be seen from table 3, no losses were found during pasteurization either by the batch, holder process, or by the continuous short-time, high-temperature process which included a deaeration step as developed at Cornell (Sharp, Guthrie and Hand, '40). There were no losses when a sample of pasteurized milk was exposed for 2 hours in a half-pint bottle to February noonday sunlight when

TABLE 3

Stability of vitamins in cow's milk.

	TREATMENT					
	Batch, holder pasteurization		Continuous short-time, high-temperature pasteurization			2 hours' exposure to sunlight
	1	2	1	2	3	
Nicotinic acid, μg per ml						
Before treatment	0.65	0.61	0.76	0.80	0.67	0.75
After treatment	0.64	0.64	0.80	0.82	0.70	0.76
Biotin, μg per liter						
Before treatment	22	21	25	24		24
After treatment	24	20	25	23		24
Calcium pantothenate, μg per ml						
Before treatment	2.3	3.6	2.6	3.5	3.4	4.2
After treatment	2.1	3.6	2.8	3.9	3.6	4.0

the temperature in the sun was 9°C. The nicotinic acid content did not decrease during at least 21 weeks' storage in the frozen state at about — 14°C., and biotin was stable for at least 19 weeks under these conditions. No nicotinic acid was lost during 24 hours' incubation of milk at 37°C. under toluene (when diluted ten-fold with water).

The beneficial effect of milk in the pellagra-preventative diets used by early workers (Goldberger and Tanner, '24) was probably due, in part at least, to its aid in alleviating coexisting deficiencies of vitamins other than nicotinic acid, since the

nicotinic acid content of milk appears to be relatively small. However, it seemed worthwhile to investigate the possibility of the presence in milk of substances similar to those found in some materials by Krehl and Strong ('44) which become active with respect to nicotinic acid assay on treatment with alkali or strong acid. No increase in the assay value of a milk sample was found when it was incubated for 3 hours at pH 10 at 37°C.

DISCUSSION

The values found for the nicotinic acid content of milk in this study were lower than those reported by most other workers. From the literature, it would seem that a typical value would be in the neighborhood of 0.8 to 0.9 μg per ml, while the nicotinic acid level of milk samples in this study averaged about 0.61 μg per ml. This mean value was for Holstein milk, and the breed comparison, while probably not very significant, suggested that the milk of other breeds has less nicotinic acid than does Holstein milk. We are not inclined to question the accuracy of the figures of others, or of our own, since the assay method is so entirely satisfactory. The individual variation between cows (see page 79) seems the most likely explanation for somewhat low values, especially since, among eight samples of mixed market milks assayed during the preliminary study of the method, the range of values found was from 0.8 to 1.04 μg per ml with a mean value of 0.9. The bulk of this study concerned a large number of samples from a small number of cows, while values usually given in the literature have been for very few samples of market milk each representing a comparatively large number of cows. The average calcium pantothenate level for all milk samples was about 3.5 μg per ml, a value fairly typical of those reported heretofore. The average biotin level was about 20 μg per liter. The individual cows dealt with in this study may have been poor biotin producers, as six samples of mixed market milk ranged from 20 to 29 μg per liter and averaged 25 μg per liter.

The great variability among individual cows in the biotin content of the milk was very surprising, especially as it seems probable that a major source of this vitamin is rumen synthesis. Various workers (e.g., Lardinois et al., '44) have reported such synthesis. It is interesting that riboflavin in milk has also been found to show much variation, both between individual cows (Hand and Sharp, '39; Johnson, Maynard and Loosli, '41; Theophilus and Stamberg, '45), and from day to day from a single cow (Riddell, '37; Whitnah, Kunerth and Kramer, '38).

The seasonal change in the nicotinic acid level of the milk seems to be at least partly correlated with pasture feeding. No sudden marked changes occurred when cows went on or off pasture, but a trend in the direction of decreasing values was reversed when pasture feeding began, and again set in, when it was stopped. It seems likely that the effect of pasturage is not due to its nicotinic acid content. Nicotinic acid has been found to be among the B vitamins synthesized in the bovine rumen (Wegner et al., '40; Lardinois et al., '44), but changes in the rumen flora as a result of going on pasture would be expected to take place much more quickly, than the changes in nicotinic acid level revealed here.

There seems to be no doubt that the nicotinic acid, biotin, and pantothenic acid originally present in the milk, remains present and available to the consumer in the milk he purchases. The stability of these vitamins in milk is in line with the results of Hodson ('45) who found no losses in the preparation of irradiated evaporated milk and of dried milks, except for a possible small loss in biotin in dried skim milk.

SUMMARY

Modifications of the usual assay methods for nicotinic acid, biotin, and pantothenic acid, which were introduced in applying them to cow's milk samples, have been described. There was no evidence that a bound form of any of these vitamins exists in milk.

The biotin levels showed great variation among the milks of individual cows. Individual averages for eight cows ranged from 11 to 37 μg per liter. Between samples obtained from the same cow on successive days, the variation was also large. These variations were less marked in the case of nicotinic and pantothenic acids.

The nicotinic acid content was found to decrease during the winter and early spring months, and to increase during the summer and early fall months. The range of values on May 9 for eight cows was 0.22 to 0.77 μg per ml. On October 16 for five cows it was 0.56 to 0.96 μg per ml. A possible correlation with pasture feeding has been suggested. The biotin content had no apparent relation to the season.

The concentration of nicotinic acid in the milk decreased regularly through much of the lactation period. The pantothenic acid content of the colostrum at the time of freshening was found to be relatively low, the average level being about 1.5 μg calcium pantothenate per ml. The mean then rose during the next 9 days to a maximum of about 4 μg per ml, and then dropped to a normal level at about 3.5 μg per ml. A similar, though earlier, rise to a maximum and fall to normal tends to be true of the biotin level of milk.

The pantothenic acid and biotin contents of milk are correlated, though the nicotinic acid level appeared not to be correlated with either.

All three vitamins were found to be stable during pasteurization and exposure of the milk to sunlight.

ACKNOWLEDGMENT

We wish to express our appreciation for the help given us in this study by Dr. J. K. Loosli. The milk samples were obtained under his direction, and we have had his full cooperation in furnishing us with all necessary data pertaining to them.

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THE EFFECT OF LIVER EXTRACTS ON THE UTILIZATION OF CASEIN FOR GROWTH [1]

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THREE FIGURES

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Liver has been shown to contain a factor, or factors, that has an accelerating effect upon the growth rate of rats and mice (Troescher-Elam and Evans, '41; McIntire et al., '43; Bosshardt et al., '45b). It has been suggested that a liver fraction may be necessary in order to obtain maximal utilization of certain dietary proteins in the growing rat. The results of Hayward and Hafner ('45) and Barnes ('45) showed an increase in the protein efficiency of a soyflour when Wilson's 1:20 liver concentrate or a butyl alcohol extract of this concentrate was included in the diet. No enhancement was noted when casein was used as the test-protein. Both proteins were fed at a level of approximately 10% in the diet.

The basal diet used by Bosshardt et al. ('45c) for the determination of the growth promoting quality of proteins included 1% of Wilson's 1:20 liver concentrate powder as an additional vitamin supplement. Since the liver concentrate powder contains 7 to 8% nitrogen, it supplies an appreciable amount of non-test nitrogen to the diet. This may introduce a serious error when low levels of test protein are used. The present study was designed to investigate the

[1] A preliminary report of this investigation was presented before the Philadelphia section of the American Chemical Society June 13, 1945.

effect of 1:20 liver concentrate and a butyl alcohol extract of this concentrate on the utilization of dietary protein by the growing mouse.

EXPERIMENTAL METHOD

The utilization of dietary protein for growth was determined in the manner described by Bosshardt et al. ('45c). Male weanling mice [2] were used. They were selected after a 2-day standardizing period during which they were fed a purified diet that contained 15% extracted whole egg. During the experimental periods, the animals were housed in individual wire-bottom cages and were supplied food and water ad libitum. Blotting paper was placed below the cages to facilitate the recovery of spilled food and the collection of feces with a minimum of urine contamination. All studies were of 20 days duration. Daily measurements were made of weight changes and food consumption. The feces of each test-group were collected for the entire test-period and "true digestibilities" were determined by the method described by Bosshardt and Barnes ('45a). Body protein gains were determined by analyses for carcass nitrogen.

Eight levels of casein ranging from 3% to 40% were studied, using groups of seven mice with each three accessory vitamin supplementations: control with no supplement, 1% of Wilson's 1:20 liver concentrate powder, and 0.15% of a butyl alcohol extract of Wilson's 1:20 liver concentrate powder prepared according to the method of Conger and Elvehjem ('41) (equivalent to 1.2% of 1:20 powder).[3] The remainder of each diet consisted of 25% hydrogenated cottonseed oil,[4] 2% corn oil,[5] 20% dextrose,[6] 4% salt mixture (Hubbell, Mendel and Wakeman, '37), 2% cellu flour, and sufficient white dextrin to make 100%. To each 100 gm of diet were added:

[2] Sharp and Dohme, Swiss-Webster strain.

[3] We are grateful to Dr. C. E. Graham of the Wilson Laboratories who supplied this extract.

[4] Primex.

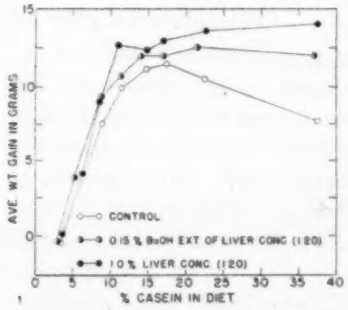
[5] Mazola.

[6] Cerelose.

4 mg of alpha-tocopherol, 900 U.S.P. units of vitamin A, 180 U.S.P. units of vitamin D, 1 mg of 2-methyl-1, 4-naphthoquinone diacetate, 0.8 mg of thiamine hydrochloride, 1.6 mg of riboflavin, 0.8 mg of pyridoxine hydrochloride, 4.0 mg of niacin, 4.4 mg of calcium pantothenate, 4.0 mg of para-aminobenzoic acid, 21.6 mg of inositol, and 200 mg of choline chloride.

The effect on growth

The average weight gains of the mice fed varying amounts of casein with the three supplementations for the 20-day test-periods are illustrated in figure 1. These data confirm the



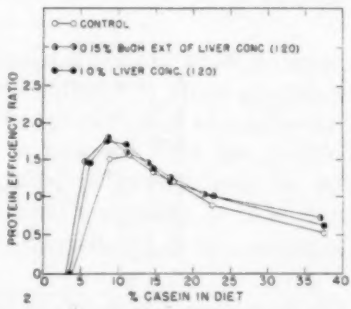


Fig. 1 The average 20-day weight gains of mice fed purified diets containing varying levels of casein with the three vitamin supplementations.

Fig. 2 The average protein efficiency ratios at 20 days determined with mice receiving purified diets containing varying levels of casein with the three vitamin supplementations.

reports previously mentioned that the addition of liver extract had an accelerating effect on the growth rate. This effect became more pronounced as the level of protein in the diet was increased.

The effect of the butyl alcohol extract was somewhat less marked than that of the original 1:20 liver concentrate. This may have been due to an incomplete extraction of the growth-accelerating factor by the butyl alcohol from the 1:20 liver concentrate.

The effect on protein utilization

Figure 2 illustrates the relationship between the level of casein in the diet and the protein efficiency ratio (gm gain in weight per gm of protein consumed) at the conclusion of a 20-day growth study. At the higher levels of protein, the protein efficiency ratio was not markedly increased by the two liver preparations, but it was consistently observed that maximal protein utilization was enhanced by both of these liver fractions. Additional studies have indicated that the protein efficiency ratio of casein is enhanced also by dried whole liver, defatted hog liver, and Valentine's [7] 1:50 liver concentrate. Data as presented in figure 2 are difficult to evaluate

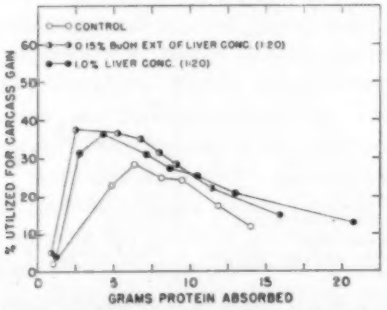


Fig. 3 The average percentage of absorbed protein utilized for body protein gain by mice during a 20-day growth study with varying intakes of casein with the three vitamin supplementations.

since no allowance is made for the differences in food consumption. Another possible source of error may be in the use of body weight gain as an index of body protein gain.

A more valid expression of the utilization of dietary protein for growth is the utilization of absorbed protein for body protein gain. This calculation was made as previously described by Bosshardt et al. ('45c). The results are shown in figure 3. A marked enhancement of protein utilization for growth by the two liver fractions was found at all levels

[7] Valentine Meat Juice Co., Richmond, Va.

of protein intake, suggesting that liver contains a factor, or factors, of unknown composition that is essential for the maximal growth utilization of casein.

DISCUSSION

The data presented in a previous communication (Bosshardt et al., '45c) indicated that such factors as the pre-test standardization of the test animals, the duration of the feeding period, and the level of the test protein in the diet may have an influence on protein efficiency ratios. The results of the present study suggest an additional factor, dietary supplementation, that may affect the utilization of protein by the growing animal.

A purified diet containing only the accessory growth factors that are recognized as chemical entities will allow growth of mice. As previously mentioned this growth may be accelerated if a liver or pancreas fraction is included with the purified vitamin mixture. This enhancement of growth may be due either to a stimulation of the animal's appetite or to a more efficient utilization of the dietary ingredients or to a combination of both.

When a single level of protein in the diet is employed, differences in food consumption and growth are not considered adequately in the calculation of the protein efficiency ratio. Such differences are minimized if protein utilization is expressed either as the maximal ratio of body weight gained to protein consumed or as the maximal ratio of body protein gained to protein absorbed. The inability of other investigators to note an increase in the protein efficiency of casein with the inclusion of liver extracts in the diet may have been due to the fact that single levels of protein in the diet were compared and maximal ratios were not established. Furthermore, as is seen in figure 3, the effect of the liver extracts is more marked when protein utilization is expressed as body protein gain rather than as body weight gain. In fact at the level of the maximal ratio the inclusion of the liver extracts

resulted in an increase of approximately 30% in the protein utilization for growth.

It was found that the rate of growth as measured by body weight gain was enhanced by both liver preparations, but the effect was more marked in the case of the untreated preparation (Wilson's 1:20). In addition there was a definite increase in the utilization of the dietary protein for growth as measured both by the gm gain in body weight per gm of protein ingested and by the percentage utilization of absorbed protein for body protein gain. The two types of liver supplement appeared to be essentially equivalent in their enhancement of protein utilization as measured by these two methods. This study demonstrates the ability of liver extracts to increase the rate of growth and to increase the utilization of protein for body protein gain. The observation that these two functions were not affected to the same extent by the two types of liver extract suggests that two factors may have been involved.

SUMMARY

Two liver fractions, Wilson's 1:20 powder and a butyl alcohol extract of the 1:20 powder, have been shown to contain a factor, or factors, that enhances the growth rate and the growth utilization of dietary protein in mice. The effect on protein utilization is demonstrated most clearly when the maximal ratios of body protein gained to protein absorbed are compared.

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THE USE OF THE DOG FOR STUDIES ON IRON AVAILABILITY [1]

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TWO FIGURES

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Dietary studies on the relation between the antianemic potency of various foods and their iron content when fed in the presence of adequate copper, have been made by numerous workers. The rat has been used almost exclusively in these investigations and consequently the applicability of these results to human nutrition may be open to some question. Since clinical investigations of this type are difficult, it follows that the work should be repeated with other species.

The dog was chosen for this study, and attempts were made to determine the availability of iron in several foods for the dog, and to determine the differences if any that exist between these values and those reported for the rat.

METHODS

Our first problem was to determine the minimal level at which the iron and iron containing foods should be fed to produce optimal hemoglobin formation. This would prevent the accumulation of large quantities of iron in the various tissues of the animal.

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To determine this level of iron, a litter of 5 collie puppies (1–6) and later a litter of 5 spaniel puppies (7–11) were placed on experiment. As in previous iron and copper work (Maass et al., '44) a diet of raw whole milk supplemented with vitamins and minerals, was fed ad libitum. That this diet is complete for the dog was demonstrated by Potter et al. ('38) who maintained dogs for long periods of time on a whole milk ration supplemented only with vitamins A and D together with iron, copper and manganese. If the proper precautions are taken against contamination, the amount of iron supplied by the diet is found to be very low (Frost et al., '40a). However, as a safeguard, the iron content of the milk fed was determined at frequent intervals (Ruegamer et al., '45).

Iron was supplied as ferric pyrophosphate.[2] This iron salt was selected because it is stable in solution and the iron is completely available. This salt together with the other mineral supplements, was added to a small amount of milk at each morning feeding.

Blood for analysis was removed from the radial vein, all samples being collected before the morning feeding. The external jugular and later the saphenous artery were used for phlebotomy when it became necessary to render the animals anemic. From 25 to 45% of the total blood volume (calculated as 8% body weight) was removed at each bleeding without apparent injury to the animals. One animal was later sacrificed for histological study and only mild hyperplasia of the bone marrow was noted.

Records were kept of the growth, the hemoglobin levels and the amount of iron fed. From these data, the total hemoglobin in the dog, hemoglobin made, iron used and the percentage of iron utilized were calculated (Frost et al., '40a). The percentage of availability of the iron in the test material can be calculated by assuming the inorganic iron to be 100% available. It should be noted that the iron furnished by the milk was not considered in the availability calculations since this factor remained constant throughout the experiment. In all

[2] Mallinckrodt, N. F. VI.

calculations, the total blood volume was considered to be 8% of the body weight. As shown by Hahn et al. ('42), the total blood volume of the dog is maintained at a constant level independent of the state of anemia.

The amount of plasma iron was determined routinely by the improved method of Kitzes et al. ('44) in an attempt to correlate the plasma iron levels with hemoglobin formation. Moore et al. ('37) state that iron is transported as plasma iron and that the quantity present in the peripheral blood is influenced by and is a measure of the amount of iron being absorbed from the gastro-intestinal tract, the iron reserve and various other factors.

RESULTS

The positive control (dog 1) from the litter of collie puppies was given 3 mg of iron per kg of body weight per day in addition to the regular mineral supplements and showed normal growth and hemoglobin levels throughout the experimental period. The remaining 5 dogs which were placed on the experimental diet at weaning, developed a severe anemia (Hb 2.1 to 3.8 gm %) in 4 to 5 weeks. At the end of the fifth week, dog 2 was given 200 μg, dogs 3 and 4 400 μg, and dogs 5 and 6 800 μg of iron per kg of body weight per day. At the end of the first week of iron therapy, dogs 2 and 3 showed no hemoglobin response. Therefore, the level of iron for these animals was raised to 600 μg of iron per kg of body weight per day. A rapid hemoglobin response occurred, suggesting that 600 μg might be adequate for growing dogs. Dog 4 continued to make hemoglobin throughout the entire experiment even though confined to the lower level of 400 μg of iron. Dogs 5 and 6, which were kept on 800 μg, continued to make hemoglobin rapidly, showing a hemoglobin increase from 4.2 and 2.1 at the start of the experiment to 9.1 and 7.5 gm % at the end of the 6-week period. To determine whether the 600 or the 800 μg level was most efficient for hemoglobin building, the per cent utilization for the iron was calculated and the results tabulated in table 1. At the end of the 6-week period,

the iron level of dogs 2 and 3 was raised to 1000 μg per kg of body weight per day. The per cent utilization for this level may also be found in table 1.

It was found that at a level of 600 μg of iron per kg per day (dogs 2 and 3) the utilization of the iron supplied as ferric pyrophosphate for hemoglobin building was 60 to 71%. Hemoglobin curves for the animals (dogs 2 and 3) receiving 600 μg of iron per kg of body weight per day showed approximately

TABLE 1

Utilization of ferric pyrophosphate for hemoglobin building over a 6-week period.

DOG NO.	LEVEL FED	IRON USED	IRON FED	IRON
	μg/kg/day	*mg*	*mg*	*%*
2	600	127	212	60
2	1000	210	541	39
3	600	134	189	71
3	1000	161	474	34
4	400	127	173	74
5	800	272	379	72
6	800	166	269	61
7	600	330	483	69
9	600	254	502	51
10	600	233	358	65
11	600	234	415	56

the same slope as that for the positive control (dog 1), receiving 3 mg of iron per kg per day (fig. 1). Levels of 800 μg (dogs 5 and 6) gave utilization of iron varying from 61 to 72% and levels of 1000 μg (dogs 2 and 3) caused the utilization to drop to 39 to 34% as would be expected. Dog 4 averaged 74% utilization on a level of 400 μg even though dog 3 failed to make hemoglobin at this level. Therefore, it can be concluded that the level of iron necessary for optimal hemoglobin building falls between 600 and 800 μg per kg of body weight per day.

From the data as plotted in figure 1, there appears to be a definite relationship between the intake of iron and the amount of iron in the plasma. When iron in excess of that

required for optimal hemoglobin formation is fed, the amount of iron in the plasma is increased. Thus when levels exceeding 600 μg of iron are fed, "normal" plasma iron values of 100–200 μg of iron per 100 ml plasma are found, and when levels below 600 μg of iron are fed, the plasma iron level drops to and sometimes below the critical level of 50 μg of iron per 100 ml of plasma. Since hemoglobin formation is greatly reduced when less than 600 μg of iron are fed, it may be concluded

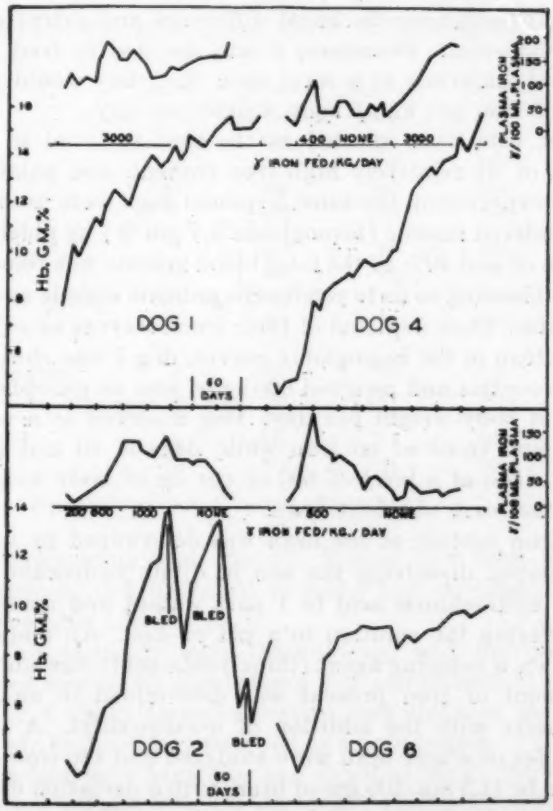

Fig. 1 Hemoglobin and plasma iron curves for dogs receiving different levels of iron as pyrophosphate.

that hemoglobin production is limited when the plasma iron level drops below 50 μg per 100 ml of plasma.

To verify our original conclusions, dogs 7–11 with the exception of dog 8 which served as a negative control, were given 600 μg of iron per kg of body weight per day after having been made anemic on a whole milk ration. At the end of 6 weeks, it was found that the animals were utilizing between 51 and 68% of the iron for hemoglobin building (table 1). These results agree with those obtained with the first litter of dogs, considering the breed difference and extent of individual variation. Therefore, it was decided to feed several biological materials at a level such that they would furnish 600 μg of iron per kg of body weight per day.

Wheat bran was selected as the first material to be fed because of its relatively high iron content, and palatability. For the experiment, the same 5 spaniel dogs were used. They were rendered anemic (hemoglobin 5–7 gm %) by phlebotomy. Between 30 and 40% of the total blood volume was removed at a single bleeding so as to render the animals anemic as quickly as possible. Once depleted of their iron reserves as evidenced by a plateau in the hemoglobin curves, dog 7 was chosen as a positive control and received 600 μg of iron as pyrophosphate per kg of body weight per day. Dog 8 served as a negative control and received no iron while dogs 9, 10 and 11 each received iron at a level of 600 μg per kg of body weight per day in the form of wheat bran.

The iron content of the bran was determined by ashing a 1 gm sample, dissolving the ash in dilute hydrochloric acid (1 part hydrochloric acid to 1 part water) and neutralizing and buffering the solution to a pH of 4.58. An aliquot was withdrawn, a reducing agent (thioglycolic acid) was added, and the amount of iron present was determined in an Evelyn Colorimeter with the addition of α-α-dipyridyl. A total of 16 samples of wheat bran were analyzed and the iron content found to be 11.5 mg/100 gm of bran, with a deviation of ± 2%. Since the animals weighed between 13 and 17 kg, between 70 and 100 gm of bran were fed daily to each dog. This supple-

ment was mixed with a little milk at each morning feeding, and the dogs were watched closely until the mixture was consumed.

. At the end of the 6-week period, it was found that the utilization of iron as supplied by the bran and as fed as pyrophosphate, were approximately the same (table 2). Likewise, the slopes of the hemoglobin curves for dogs receiving bran (9, 10 and 11) were equivalent to the slope of the curve for

TABLE 2

Iron utilization for dogs receiving bran, spinach or ferric pyrophosphate as a source of iron over a 6-week period.

SOURCE	DOG NO.	LEVEL FED	IRON USED	IRON FED	IRON UTILIZED
		µg/kg/day	*mg*	*mg*	%
Bran	7[1]	600	209	406	51
	9	600	259	385	68
	10	600	209	330	69
	11	600	259	429	60
Spinach	7	600	30	266	11
	9	600	51	274	18
	10	600	41	216	19
	11[1]	600	134	260	51
Ferric pyrophosphate	7	600	303	516	59
	8	600	295	458	65
	9	600	248	365	68
	10	600	214	409	52
	11	600	151	337	45

[1] Control dogs receiving iron as ferric pyrophosphate.

the dog receiving iron (fig. 2). During this time, the negative control (dog 8) failed to make any significant amount of hemoglobin.

The supplements were discontinued and the dogs were rendered anemic by phlebotomy and maintained at this level for 3 or 4 weeks to make certain that the iron stores were depleted. Spinach was then fed as a supplement. The spinach was washed very carefully and dried at 46°C. by passing a stream of hot air over the material. The dried spinach was ground and the iron content determined as in the case of the

bran. 20.7 mg of iron per 100 gm of dried material were found, thus necessitating the feeding of approximately 40–50 gm of spinach to each dog daily. However, since the spinach proved to be unpalatable, it became necessary to mix the spinach with a small amount of milk at each feeding and give it by stomach tube. In this experiment, dog 11 received inorganic iron and served as the positive control and dogs 7,

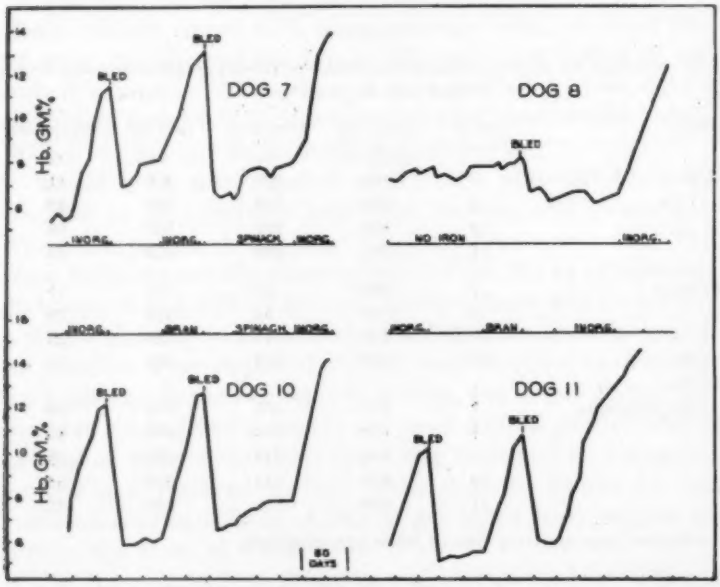

Fig. 2. Hemoglobin curves for dogs receiving iron as wheat bran, spinach and pyrophosphate.

9 and 10 received the spinach. Dog 8 served as the negative control as before. At the end of a 6-week period it was found that the spinach dogs (7, 9 and 10) utilized only 10 to 20% of the iron in the spinach for hemoglobin building, whereas the positive control (dog 11) utilized 50% (table 2). As can be seen from figure 2, there is also a marked difference in the slopes of the curves showing hemoglobin regeneration.

To determine if phlebotomy had had any effect on the blood building mechanism, all animals were given iron as pyrophosphate at a level of 600 μg per kg of body weight per day. As before, the animals utilized between 52 and 68% of the iron for hematopoiesis (table 2).

DISCUSSION

The results of this experiment indicate that levels of 600 to 800 μg of iron per kg of body weight per day are optimal for hemoglobin formation in the dog. Breed difference seems to have little or no effect on the iron requirement since it was possible to obtain essentially the same results in two breeds of dogs. If less than 600 μg of iron are fed, the animals will fail to make hemoglobin, and if more than 800 μg are fed, the per cent utilization will decrease. Therefore, since storage of iron in the tissues is to be avoided, the test materials were fed at a level such that 600 μg of iron were supplied per kg of body weight per day. That little storage of iron took place is shown by the hemoglobin curves (fig. 2). In each case, when rendered anemic, usually by one bleeding, the animal failed to make hemoglobin until iron supplementation was started.

The iron in bran was found to be almost completely available for hemoglobin formation. Of course, there is the possibility that additional factors supplied by the bran stimulated hemoglobin production. This seems unlikely however, since it has been demonstrated that milk will support hematopoiesis in dogs without the addition of factors other than iron, copper and manganese (Maass et al., '44; Frost et al., '40a, b).

When spinach was fed as the sole source of iron, very poor hemoglobin regeneration occurred. The per cent utilization of the spinach iron averaged between 10 and 20% as compared to 50% for the iron given as ferric pyrophosphate. As mentioned previously, the spinach was dried at 46°C. to simplify the feeding problem and this processing might possibly have had some effect on the availability of the iron, since it has

been found that refrigeration, for example, will increase the availability of the iron in spinach (Hastings et al., '41). Under the conditions of this experiment, however, we found the availability of iron in spinach to be very poor.

Little iron contamination of the basal milk ration occurred as evidenced by the failure of the negative control to produce significant amounts of hemoglobin, and by the frequent assays of milk fed. However, one animal (dog 4) did make hemoglobin on the low level of 400 μg of iron per kg of body weight per day, and thus it is possible that this animal obtained iron from outside sources either from the cage or through handling.

When the values for the availability of iron in bran and spinach as obtained with this assay method on dogs are compared to those obtained with rats, a close correlation is found. Recent work in this laboratory with the rat has shown that the iron in bran is completely available as compared to ferric pyrophosphate (unpublished data). Sherman et al. ('34) found the iron in spinach as assayed by the α-α-bipyridine method and also the rat biological method to be only 20% available.

SUMMARY

Young growing dogs were placed on a raw whole milk ration, supplemented with vitamins, copper and manganese. When the dogs were anemic, supplements of ferric pyrophosphate at levels ranging from 200 μg to 1000 μg of iron per kg of body weight per day were supplied. A minimal level of 600 μg of iron was found to give an optimal hemoglobin response.

Plasma iron levels were followed throughout the period of iron supplementation, and it was found that when iron in excess of that required for optimal hemoglobin formation was fed, the amount of iron in the plasma increased. If suboptimal amounts of iron were fed, the plasma iron level dropped to and sometimes below the critical level of 50 μg of iron per 100 ml plasma.

Wheat bran and spinach were fed at a level to supply 600 μg of iron per kg of body weight per day, and the response compared with that obtained with ferric pyrophosphate. The iron in bran was found to be almost completely available while the iron in spinach was only 20–40% available.

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SELF SELECTION OF DIET

II. THE EFFECT OF FLAVOR [1]

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From human experience, one could conclude that choice of food is to a large extent determined by flavor, and thus flavor of food may be important in determining nutritional status. Further, it has been often assumed that if animals refuse to eat a particular food, the taste of that food is unpleasant to the animals and a more pleasing flavor would be preferred. Hausmann ('33) reported that rats preferred to drink water flavored with saccharine to pure water. Jukes ('38) found that chickens avoided diets containing more than 2% salt, 0.03% quinine or 2% citric acid. Diets to which diacetyl was added were preferred by rats, according to Deuel and Movitt ('44).

The present experiments were designed to determine the importance of certain flavors in the self selection of diet by rats. A method of determining dietary preference for a single factor is described which is adaptable to a wide variety of dietary components.

METHODS

In the first experiment here reported, the eating behavior of animals allowed to choose from two identical diets was determined over a 6-week period to serve as a control test

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of the method. Ten male and ten female rats were weaned at 21–25 days of age. Each rat was placed alone in a cage with a rack containing two cups filled with a standard diet.[2] Vitamins were supplied separately as pills.[3] The amount of food eaten from each cup was recorded daily and the cups were then alternated in a predetermined random sequence.

In testing for appetites for flavors, a small amount of flavor was added to the standard diet. The flavors used were diacetyl, 3 p.p.m.; oil of anise, 40 p.p.m.; monosodium glutamate, 1%[4]; butyric acid, 600 p.p.m., and trimethylamine in a not readily measurable amount.[5] The flavors of the diets containing diacetyl and monosodium glutamate were quite mild, while the other three were offensively strong by human standards.

Ten weanling animals in each flavor test (five males and five females) were given the unflavored standard diet during a 3-week period in both cups, while another ten were given the flavored diet in both cups. At the end of this control period, both groups were allowed their choice of the standard and flavored diets for another 3 weeks. The amount of food eaten from each cup was recorded daily during both periods, and the cups alternated in the same predetermined random manner.

RESULTS

The results of the control experiment are shown in table 1. The 6-week period was divided in half to conform to the conditions of the other experiments, but very little difference

[2] The standard diet consisted of 24% purified casein (Labco "Vitamin-Free"), 10% hydrogenated fat (Primex), 4% salts (Jones and Foster, '42), and 62% sucrose.

[3] Each pill contained approximately: 60 μg thiamine hydrochloride; 120 μg riboflavin; 90 μg pyridoxine hydrochloride; 150 μg calcium pantothenate; 10 mg choline chloride; 1 mg α-tocopherol and 55 I.U. vitamin A and 11 I.U. vitamin D as 0.001 ml Natola in a dextrin-powdered sugar base. One pill was fed to each animal daily.

[4] The diet containing 1% monosodium glutamate contained 23% casein.

[5] Trimethylamine gas was dissolved in a few ml of water, and the solution added to the diet until a strong fishy odor was apparent.

TABLE 1

Eating behavior of rats when no choice is offered.

	FIRST 3 WEEKS [1]				SECOND 3 WEEKS			
	Wt. gain	Total food eaten	% of food from		Wt. gain	Total food eaten	% of food from	
			Left cup	Cup I [2]			Left cup	Cup I
	gm	*gm*			*gm*	*gm*		
Males	59.2 ± 4.4	138.5 ± 9.4	48.1 ± 3.6	48.8 ± 3.1	61.4 ± 4.5	222.7 ± 16.2	49.4 ± 2.8	43.4 ± 4.0
Females	47.0 ± 2.4	123.0 ± 6.2	47.4 ± 2.4	49.5 ± 1.5	41.4 ± 2.3	184.7 ± 6.8	53.7 ± 4.4	54.4 ± 2.0

[1] All data in terms of mean and standard error of the mean.

[2] Cup I was alternated between the left and right positions.

TABLE 2

Effect of diacetyl flavor on selection.

	CONTROL PERIOD				EXPERIMENTAL PERIOD				
Diet	Wt. gain	Total food eaten	% of food from		Diet	Wt. gain	Total food eaten	% of food from	
			Left cup	Cup I [1]				Left cup	Cup I
	gm	*gm*				*gm*	*gm*		
Group A Standard	51.1 ± 3.5	122.7 ± 5.3	53.7 ± 5.5	48.2 ± 7.2	Choice	64.8 ± 5.7	206.6 ± 10.9	50.9 ± 3.1	46.3 ± 8.6
Gruop B Flavored	58.3 ± 3.6	133.9 ± 7.3	43.5 ± 2.9	52.3 ± 3.6	Choice	65.5 ± 6.1	202.8 ± 14.2	44.2 ± 1.8	44.1 ± 5.2

	Change in % eaten (control minus experimental)	
	From left cup	From cup I
Group A	2.8 ± 5.2	1.9 ± 11.5
Group B	− 3.7 ± 4.0	8.2 ± 10.1
All	− 0.4 ± 3.3	5.0 ± 7.5

[1] Cup I contained the standard diet in the experimental period; cup II the diacetyl-flavored diet. A positive change in per cent eaten from cup I (control minus experimental) therefore represents a preference for the flavor.

TABLE 3

Effect of flavors on selection.

		CONTROL PERIOD		EXPERIMENTAL PERIOD			CHANGE IN % EATEN
	Diet	Wt. gain	Total food eaten	Diet	Wt. gain	Total food eaten	From cup I (Control minus experimental)
		gm	*gm*		*gm*	*gm*	
				Anise			
Group A	Standard	75.2 ± 5.7	154.6 ± 10.4	Choice	75.0 ± 7.6	259.7 ± 15.4	− 1.6 ± 8.3
Group B	Flavored	70.0 ± 6.5	144.8 ± 11.9	Choice	70.4 ± 8.6	242.2 ± 21.6	− 5.1 ± 5.1
All							− 3.4 ± 6.9
				Trimethylamine			
Group A	Standard	63.9 ± 1.9	148.6 ± 3.0	Choice	41.0 ± 2.5	197.4 ± 6.9	− 20.9 ± 5.9
Group B	Flavored	63.2 ± 2.2	156.7 ± 3.8	Choice	41.2 ± 3.5	207.6 ± 4.5	9.9 ± 14.1
All							− 5.5 ± 6.4
				Monosodium glutamate			
Group A	Standard	81.4 ± 1.1	181.0 ± 7.2	Choice	65.4 ± 7.9	266.0 ± 11.7	7.3 ± 4.9
Group B	Flavored	79.7 ± 3.3	180.2 ± 6.7	Choice	57.5 ± 7.9	256.0 ± 9.3	− 0.5 ± 5.5
All							3.4 ± 2.6
				Butyric acid			
Group A	Standard	74.0 ± 4.7	163.4 ± 7.6	Choice	73.2 ± 6.2	252.4 ± 11.7	− 12.3 ± 9.3
Group B	Flavored	72.2 ± 4.2	173.0 ± 8.7	Choice	71.0 ± 4.7	261.8 ± 11.0	− 0.8 ± 4.3
All							− 6.6 ± 5.7

was observed in the two periods. Table 2 presents in detail the results of the experiment on diacetyl. The results with other flavors are shown in condensed form in table 3. The animals as a whole showed neither likes nor dislikes for any of the flavors, although the animals on an unflavored diet appeared to dislike trimethylamine (fishy) flavor.

DISCUSSION

The method of determination of dietary preferences described here is intended to be of general applicability. To adequately assess its usefulness, it is necessary to consider certain factors in animal behavior and mechanics of methods.

1. Position eating. In a 2-choice experiment many animals prefer to eat from either the left or right cup, and of these, a slight majority prefer the cup in the right-hand position. This habit was corrected for by alternating the cups. Since rats can learn a simple alternation pattern, the interchange was made random. The method of alternation was predetermined and was made identical in every case. In all experiments in this laboratory there have been no significant differences in the "position eating" habits of a group of rats when the control and experimental periods were compared. Tables 1 and 2 present typical cases.

2. Repetitious eating. If identical diets are offered, rats tend to eat from the cup they have eaten from before regardless of the change of position (Scott, '46). If the diets are not identical, repetitious eating by a single animal is indistinguishable from choice by a group because in repetitious eating both of the choices are eaten by the group in nearly equal amounts, while one is preferred if the group has made a choice. The present method then is valid only for demonstrating an appetite by a group.

When the diets are not identical, rats show a marked tendency to eat one diet or the other, but not both. This is indicated by the high values of the standard error of the mean of the change in per cent eaten from cup I in tables 2 and 3, and may easily be due to the fact that detectable dif-

ferences between choices in diet make repetitious eating easier. There is no evidence in these experiments that the increased tendency of individuals to eat the same diet when a choice is offered is an expression of individual preference.

3. Controls. The method of determining dietary preference presented here depends on a difference in eating behavior when a choice is or is not offered. Each animal served as its control. This was justified by the data in table 1, where no significant change in eating behavior was observed when the first and second 3-week periods were compared.

4. Previous diet. Besides determining dietary preference, this method permitted the effect of previous diet on selection to be determined, since half of the animals were given one choice and the other half the other choice during the control period. In the case where appetite for a dietary essential is studied, the effect of the previous diet includes the effect of nutritional state. In only one case in these experiments (trimethylamine) did the previous diet appear to have an influence on selection. In general, our experience has made it appear probable that the previous diet affects selection only during the first 1 or 2 days of the experimental period.

5. Sex and variation. Although both sexes were used in these experiments, no difference in appetite attributable to sex difference was found. The litters used were divided between groups A and B in every experiment to avoid any familial variation. The differences in growth rate of animals used in the several experiments are apparently due to natural variation.

6. Statistical treatment. The data obtained have been treated statistically primarily for clarity of presentation, while it was fully recognized that the number of animals used was somewhat small for analysis. Since these experiments concern phenomena which have not been well studied, it was believed that statistical treatment would disclose important general trends, if not details. Thus, experiments on a larger number of animals might show that the apparent avoidance of trimethylamine flavor by rats on a standard diet was not

real, but there can be little doubt that flavors of the type tested are in general of little importance to rats in selecting food.

While five experiments are really too few on which to base general conclusions, it seems probable that a considerable number of other aromatic and pungent flavors would give comparable results. From the growth and food intakes during the control periods in tables 2 and 3 it is apparent that none of these flavors had any dietary importance or effect on overall appetite.

Part of the usefulness of rats in nutritional research can be attributed to the fact that these animals, unlike guinea pigs and others, seldom refuse to eat an experimental diet. This, and the omnivorous habit of rats, may well be associated with the lack of importance which they attach to flavor of food.

SUMMARY

A method of determining dietary preference of rats is described. No appetite for nor avoidance of the flavors of diacetyl, anise, monosodium glutamate, or butyric acid could be demonstrated, although animals previously fed an unflavored diet appeared to avoid trimethylamine (fishy) flavor.

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Erratum

In the paper entitled "The carotene content of Cuban foods" by Angulo et al. which appeared in the April, 1946, issue, the data for carotene content should be in "μg/gm of material" instead of mg/gm of material." Please insert in table 1, column 4 (pp. 466–467) "μg" in place of "mg."